LIPASE CATALYSED ESTERIFICATION OF METHYL GLUCOPYRANOSIDE WITH SELECTED ALIPHATIC CARBOXYLIC ACIDS: INVESTIGATION ON REACTION PARAMETERS AND SURFACTANT PROPERTIES

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ABSTRACT

Carbohydrate esters are non-ionic biodegradable surfactant that possessed wide range of hydrophilic-lipophilic balance (HLB) value. It has been extensively produced *via* conventional chemical esterification route. This resulted in a number of disadvantages e.g. environmental pollution and personnel safety. Enzyme catalyzed production of carbohydrate esters is viewed as an alternative route to chemical synthesis. Lipase belongs to hydrolase, a green and safer family that has been widely utilized in synthetic steps. Conventionally, lipase is known for its capability to hydrolyze triacylglyceride in an aqueous system. On the contrary, lipase exhibits esterase activities in the micro-aqueous system.

In this study, lipase- catalyzed esterification reaction and properties of synthesized carbohydrate esters were investigated. Methyl α-*D*-glucopyranoside as acyl group acceptor and different carbon atom chain length of carboxylic acids as the acyl group donor (hexadecanoic acid, tetradecanoic acid and dodecanoic acid) were applied in the esterification. Full factorial experimental design was used to study the effects of different process parameters toward lipase-catalyzed esterification. Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was used as the model carbohydrate ester in the study. It was found *via* statistical analysis ($\alpha = 0.05$) that process parameters *viz.* substrates mol ratio (acyl acceptor: acyl donor), enzyme loading (g), quantity of 4Å molecular sieve (g), reaction time (hr) and agitation speed (rpm) significantly affected the esterification and only reaction temperature (°C) did not.

Melting point for the methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was the highest at 81 °C followed with methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside at 80 °C and the lowest was recorded by methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside at 73 °C.

Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside had melting enthalpy of 34.8 kJ mol^{-1}, 48.0 kJ mol^{-1} and at 40.9 kJ mol^{-1} respectively. For the melting entropy, the values for methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was 0.43 kJ mol^{-1} $°C^{-1}$, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside 0.60 kJ mol^{-1} $°C^{-1}$ and methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside 0.55 kJ mol^{-1} $°C^{-1}$. Liquid crystal properties of the synthesized carbohydrate ester synthesized were evaluated *via* Optical Polarized Microscopy (OPM). It was found that the liquid crystal textures for mono-substituted carbohydrate ester were of smectic phase. Evaluation on the maximum water mass contained in a quarternary system (carbohydrate ester/*n*-butanol/*n*-hexadecane/water) was done through a pseudoternary phase diagram. Phase diagram showed maximum 34% water contained in monophasic region of methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and maximum of 52% water contained in a monophasic methyl-6-*O*-dodecanoyl-α-*D*-glucopyranoside. For methyl-6-*O*-dodecanoyl-α-*D*-glucopyranoside, its concentration at aggregation was 5.2 x 10^{-4} mM, with minimum air/ water surface tension of 26 mN m^{-1}. Gibbs energy of micellization was calculated at -50 kJ mol^{-1}. Maximum adsorption density of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was determined at 4 x 10^{-6} mol m^{-2} while its mimimum area per surfactant molecule at air/ water surface was 47 $Å^2$.

ABSTRAK

Ester karbohidrat adalah salah satu contoh surfaktan yang mesra alam yang mempunyai julat keseimbangan hidrofilik-lipofilik yang besar. Pada kebiasaannya, penghasilan ester karbohidrat adalah melalui proses sintesis kimia. Proses ini walaubagaimanapun telah menyumbang kepada pencemaran alam sekitar serta. Penggunaan enzim dalam penyediaan ester karbohidrat telah dilihat sebagai proses alternatif kepada masalah ini. Enzim 'lipase' tergolong dalam kumpulan 'hydrolase' yang mana ia pada asalnya telah digunakan untuk memecahkan triasilgliserida dalam larutan air. Peranannya walaubagaimanapun berubah kepada 'esterase' apabila berada dalam sistem yang mempunyai kandungan air yang amat sedikit yang mana ia dapat menjadi pemangkin kepada proses esterefikasi.

Fokus penyelidikan ini adalah terhadap proses esterifikasi yang dimangkinkan oleh 'lipase' serta ciri-ciri fizikal ester karbohidrat yang terhasil. Metil-*D*-glukopiranosida telah digunakan sebagai penerima kumpulan asil daripada asid karbosilik dan asid karbosilik berlainan panjang atom karbon (asid heksadekanoik, asid tetradekanoik dan asid dodekanoik) telah digunakan sebagai penyumbang kumpulan asil. Rekabentuk eksperimen telah digunakan untuk mengkaji kesan pelbagai faktor-faktor proses terhadap esterifikasi yang dimangkin oleh 'lipase' dengan menggunakan perisian computer Minitab®. Berdasarkan kepada analisis statistik ($\alpha = 0.05$), hampir kesemua proses eksperimen yang digunakan iaitu nisbah mol substrat (penerima kumpulan asil : penyumbang kumpulan asil), kandungan enzim (g), kandungan penyerap air molekular bersaiz 4Å (g), tempoh eksperimen (jam) dan halaju pencampuran (rpm) mempengaruhi eksperimen dengan signifikan dan hanya suhu tindakbalas didapati tidak mempengaruhi eksperimen dengan signifikan .

Suhu pencairan bagi metil 6-*O*-heksadekanoil-α-*D*-glukopiranosida mencatatkan yang tertinggi iaitu pada 81 °C diikuti dengan metil 6-*O*-tetradekanoil-α-*D*-glukopiranosida pada 80 °C dan terendah telah direkodkan oleh metil 6-*O*-dodekanoil-α-*D*-glukopiranosida iaitu pada 73 °C. Metil 6-*O*-heksadekanoil-α-*D*-glukopiranosida mempunyai entalfi pencairan dengan 34.8 kJ mol^{-1}, metil 6-*O*-tetradekanoil-α-*D*-glukopiranosida merekodkan entalfi pencairan pada 48.0 kJ mol^{-1} dan metil 6-*O*-dodekanoil-α-*D*-glukopiranosida pada 40.9 kJ mol^{-1}. Entropi pencairan oleh metil 6-*O*-heksadekanoil-α-*D*-glukopiranosida adalah sebanyak 0.43 kJ mol^{-1} $^{o}C^{-1}$, metil 6-*O*-tetradekanoil-α-*D*-glukopiranosida sebanyak 0.60 kJ mol^{-1} $^{o}C^{-1}$ dan metil 6-*O*-dodekanoil-α-*D*-glukopiranosida sebanyak 0.55 kJ mol^{-1} $^{o}C^{-1}$. Keberadaan struktur separa kristal pada ester karbohidrat yang terhasil telah dikenalpasti dengan menggunakan mikroskop kanta polar. Didapati struktur separa kristal bagi ester karbohidrat (mono) adalah berbentuk smektik. Penyelidikan terhadap kandungan air dalam sistem kuarternari (ester karbohidrat/*n*-butanol/*n*-heksadikana/air) telah dijalankan dan melalui gambarajah pseudo-ternari yang telah dirangka, sebanyak 34% kandungan air maksimum terkandung di larutan homogen apabila metil 6-*O*-tetradekanoil-α-*D*-glukopiranosida digunakan sebagai surfaktan dan 52% kandungan air terlarut di dalam larutan homogen apabila metil 6-*O*-dodekanoil-α-*D*-glukopiranosida digunakan sebagai surfaktanmetil. 6-*O*-dodekanoil-α-*D*-glukopiranosida merekodkan kepekatan ketika agregasi pada 5.2×10^{-4} mM, dengan ketegangan antara fasa udara/air direkodkan pada 26 mN m^{-1}. Tenaga terbebas Gibbs ketika agregasi adalah -50 kJ mol^{-1}. Ketumpatan maksima lekatan yang dialami oleh metil 6-*O*-dodekanoil-α-*D*-glukopiranosida adalah 4×10^{-6} mol m^{-2} dan keluasan minima surfaktan per molekul udara/air adalah 47 $Å^2$.

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May God bless you.

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LIST OF ABBREVIATIONS

CAL B	*Candida antarctica* lipase B
Ser	Serine
His	Histidine
Asp	Aspartate
Glu	Glutamate
SDS	Sodium deodecyl sulphate
HLB	Hydrophilic lipophilic balance
CMC	Critical micellar concentration
α / β	Alpha / beta
kDa	kilo Dalton
Log *P*	Octanol – water partition coefficient
DMSO	Dimethylsulfoxide
*p*NPP	*para*- nitrophenyl palmitate
NaOH	Sodium hydroxide
ε	Molar extinction coefficient (L mol^{-1} cm^{-1})
A	Absorbance
C	Concentration (mol L^{-1})
l	Cuvette path length (1 cm)
Δ Abs_{410}	Different in absorbance at 410 nm at known time interval (min^{-1})
V_s	Total assay volume (mL)
V_{ls}	Volume of substrate (mL)
rpm	round per minute
(w/v)	weight per volume
(v/v)	volume per volume
(w/w)	weight per weight
PTFE	Polytetrafluoroethylene
TLC	Thin layer chromatography

R_f	Retention factor
NMR	Nuclear magnetic resonance
DSC	Differential scanning calorimetry
OPM	Optical polarized microscopy
UV-Vis	Ultraviolet-visible
PSA	Phenol sulphuric acid
δ	delta
% D	Deuteration (%)
TMS	Tetramethylsilane
Å	Angstrom
ANOVA	Analysis of variance
DF	Degree of freedom
SS	Sum of squares
MS	Mean square
F	F-value
P	P-value
T_m	Melting temperature
ΔH_f	Endothermic melting enthalpy
ΔS_f	Entropy of melting
CAC	Critical concentration of aggregation
γ_{cac}	Surface tension of surfactant at critical concentration of aggregation
γ_{min}	Minimum surface tension
Γ	Surface excess
ΔG_{mic}	Gibbs free energy of micellization
$d\gamma$	Change of surface tension
R	Universal gas constant
A_0	Minimum area per surfactant molecule at air/ water surface
N_A	Avogadro constant (6.023×10^{23} mol^{-1})

CHAPTER ONE

1.0 INTRODUCTION

There are numerous ways to synthesize carbohydrate esters *via* chemical routes. Chemical processes have been widely used in industries to produce products that have value added properties for daily applications. The process however, requires extreme conditions that are known to be harmful towards the environment as well as personnel's health and safety. Research on enzyme mediated reactions or biocatalysis for the production of carbohydrate esters as commercial products have been actively pursued to mitigate the hazard issues.

The enzyme lipase from *Candida antarctica* (*E.C 3.1.1.3*), which is an example of hydrolase, is the catalyst of interest to be utilized in this study. *Candida antarctica* lipase (CAL B) has been widely studied for esterifications (Arsan & Parkin, 2000; Yoo *et al.*, 2007; Zoumpanioti *et al.*, 2008). This enzyme belongs to a hydrolase family with a conserved catalytic groups of Ser, His, and Asp/Glu (Uppenberg *et al.*, 1995). Lipases in ordinary systems (aqueous) catalyze the interfacial hydrolysis of triacylglycerol to produce glycerol and fatty acids. The reaction direction, however, is reversed when lipase is being utilized in micro-aqueous environment (organic solvent), where lipase instead catalyzes an esterification process. Esterification is a process where an alcohol and a carboxylic acid combine under water elimination to form an ester, which exhibits different properties than the starting materials. A product example of an esterification (where a carbohydrate is used as acyl acceptor and fatty acid is used as acyl donor) is a carbohydrate ester. Carbohydrate esters are surfactant as the nature of the produced molecule is ampiphilic. The presence of hydrophilic and hydrophobic domains makes it tolerable to both water and oil media. Lipases are known to show high selectivity in

esterifications. The attachment of a carboxylic acid to a carbohydrate has been found to favour the carbon 6 (C_6) of the glucose (Cauglia & Canepa, 2008), thus resulting in formation of a carbohydrate monoester.

Surface-active agents (surfactants) have been extensively used in various fields, especially in cleaning industries. The structure for surfactant comprises of two molecular regions with different polarity, *i.e.* hydrophobic and hydrophilic groups, which increase the surfactant's versatility in solvation capability. The presence of these two regions confers with the ability to lower the surface tension of water and enabled the dissolution of two otherwise immiscible liquids. Surfactants are commonly used as emulsifier, i.e. in food industries. Ionic and non-ionic surfactants are different types of surfactants that have been extensively manufactured and distributed worldwide. Negatively charged surfactant (anionic surfactant) are the most widely distributed surfactant (Schmitt, 2001). They are frequently applied in households as well as in the industry. Sodium deodecyl sulphate (SDS) is the most common surfactant. It is used in various fields as essential component, e.g. in shampoos and in toothpastes. Cationic surfactants, on the other hand, are not used as general purpose detergents as they are not capable of effective cleaning at pH 7. These surfactants are widely used as fabric softeners as well as antimicrobial agents. Non-ionic surfactants are the most preferable type of surfactant due to their environmental friendliness. They tend to be more effective than other surfactants due to higher stability in the formed emulsion when applied for oil removal. Non-ionic surfactants possess a wide range of hydrophilic-lipophilic balance values ($1 < HLB < 20$), thus covering the whole range for surfactant miscibility with water and oil. Most of the non-ionic surfactants are low foaming agents and their compatibility with cationic fabric softeners makes them superior to anionic surfactants in certain formulations (Schmitt, 2001). Carbohydrate fatty acid esters are

one of the well-known non-ionic surfactants. They belong to the most preferred type of surfactant due to their biodegradability and the wide range of HLB value.

This study focuses on the synthesis of non-ionic surfactant, *viz.* carbohydrate fatty acid esters, catalyzed by a lipase. Three different chain lengths of acyl donors (fatty acid) and one specific acyl acceptor were investigated; dodecanoic acid (C-12), tetradecanoic acid (C-14) and hexadecanoic acid (C-16) were used as acyl donors while methyl α-*D*-glucopyranoside was applied as the acyl group acceptor. Besides synthesis, the thermo-chemical properties of each product were studied. The reactions were conducted in a tertiary alcohol (*tert*. butanol) as the reaction requires minimal amount of water to proceed. The product from the esterification of hexadecanoic acid and methyl α-*D*-glucopyranoside is an ester known as methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside. The attachment of acyl groups to methyl α-*D*-glucopyranoside is shown in Figure 1.1:

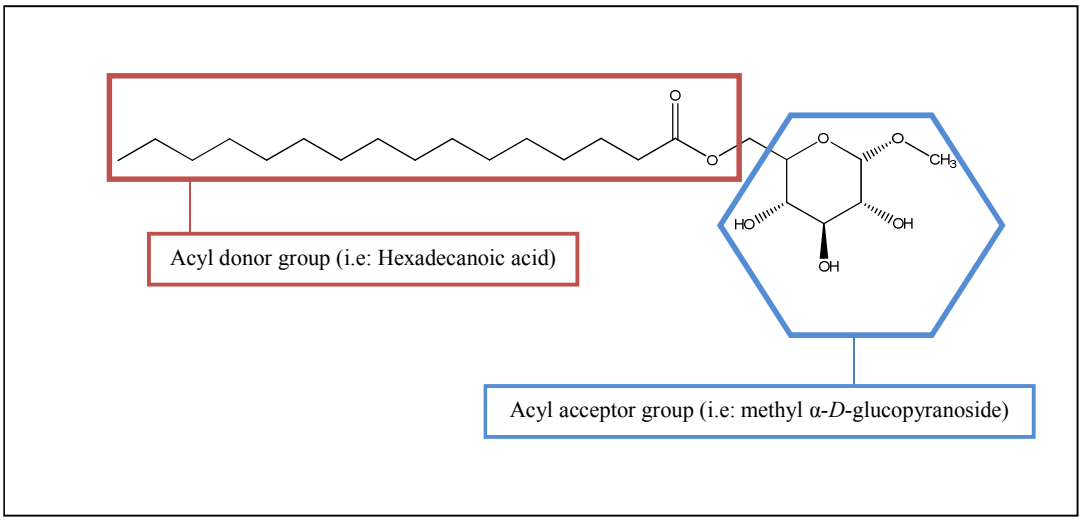


Figure 1.1: Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside

A full factorial experimental design was used in the experiment where the arrangement is based on specific statistical algorithm. Analysis of the responses is analyzed systematically. By applying full factorial experimental design in planning the experiment, an experimenter is able to investigate the effects among factors tested in the system studied. The amount of product expected to be limited due to solubility limitation of the acyl acceptor, i.e. the carbohydrate, as one of the substrates in the reaction mixture. However, in this study the presence of a methyl group at C_1 in methyl α-*D*-glucopyranoside reduced the poor solubility of glucose in organic solvents. Esters of methyl α-*D*-glucopyranoside have been known to increase the lipophilic character of the surfactant. Due to their good emulsification properties, methyl α–*D*-glucopyranoside esters have been widely used as moisturizing and emulsifying agents, as well as thickeners for cosmetics (Behler *et al.*, 2001).

The objectives of this study were:

1) To study the efficiency of *Candida antarctica* lipase B for the production of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside;
2) To study the effects of selected process parameters towards the synthesis of the carbohydrate ester;
3) To study the selected physico-chemicals properties of the synthesized carbohydrate esters;
4) To investigate the effects of different chain length of fatty acid (acyl donor) towards the thermo-chemical properties of synthesized carbohydrate esters.

CHAPTER TWO

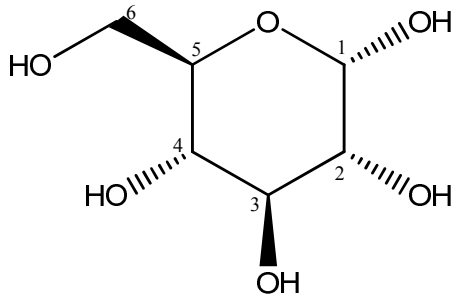
2.0 LITERATURE REVIEW

2.1 CARBOHYDRATE

Carbohydrate is a class of compound that has a general formula of $C_m(H_2O)_n$. There are several general classes in the carbohydrate family *viz.* monosaccharides, disaccharides, oligosaccharides and polysaccharides. Monosoccharide is the simplest form of carbohydrate. This form of carbohydrate is further divided into two different sub-group, polyhydroxyaldehydes (aldoses) and polyhydroxyketones (ketoses; *i.e*: fructose).

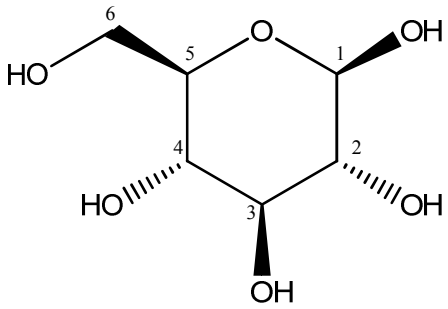
Glucose is known to be the most common carbohydrate found in nature and has a variety of uses. It comprises of six member pyranose ring and is an example of an aldose. The structures of α and β forms of *D*-glucose are shown in Figure 2.1 (a) and (b), respectively:

(a)



α –*D*- glucose

(b)



β –*D*- glucose

Figure 2.1: (a) α-*D*-glucose, (b) β -*D*- glucose

Glucose has been extensively utilized in researches as they are known to be poly-functional compounds that can undergo reactions targeting each of the other functional groups available in reactions. Due to its simplicity compared to other carbohydrates, glucose has become one of the favourite substrates (as acyl group acceptor) for esterification (Degn & Zimmermann, 2001; Flores *et al.*, 2002; Cauglia & Canepa, 2008).

Methyl α-*D*-glucopyranoside is a derivative of glucose, in which the hydroxyl (-OH) group in carbon one (C_1) is replaced by methoxy group through methylation. The structure for methyl α-*D*-glucopyranoside is shown in Figure 2.2.

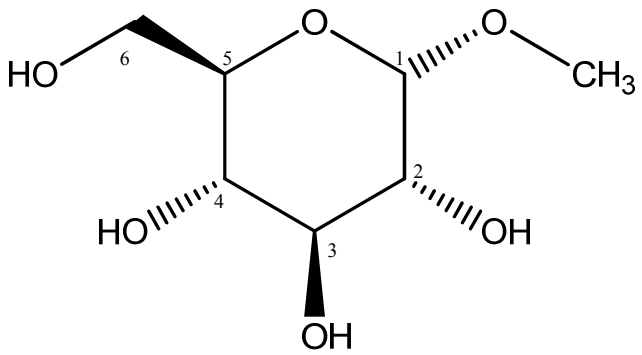


Figure 2.2: Methyl α–*D*-glucopyranoside

Due to differences in polarity between glucose and most organic solvents, the solubility of unprotected glucose is very poor. To reduce the polarity gradient between glucose and the organic solvent, the hydroxyl group at C_1 can be replaced by a methoxy group ($-OCH_3$). The presence of methyl groups at C_1 reduces the poor glucose solubility in the organic solvent. Methyl glucoside is easier to dissolve in organic solvent thus the it is easier to operate the system. Due to their good emulsification properties, methyl α-*D*-glucopyranoside esters have been widely used as moisturizing, emulsifying agents, as well as thickeners for cosmetics (Behler *et al.*, 2001).

2.2 FATTY ACIDS

Fatty acids are aliphatic carboxylic acid and known to be weak acids due to presence of carboxyl group at the end of the aliphatic chain. Generally, fatty acids can be either saturated fatty acids or non-saturated, referring to the absence or presence of double bond(s). The length of fatty acids can range from less than six up to more than 24 carbon atoms. The compounds are electrophilic molecules; due to their ability to accept electron from an electron donor group thus, they are usually applied for reactions involving electron group transfer in order to produce new products, *e.g*: esters.

Fatty acids were used as one of the substrates in either transesterification or interesterification. For example, biodiesel can be produced through transesterification (Shah *et al.*, 2003). Sugar ester on the other hand can be produced through inter-esterification (Moh *et al.*, 2000). In esterification of fatty acids, the carbon chain length of the fatty acid determines the character of the produced ester; *viz.* if a long chain fatty acid is used as the acyl (R-C=O) donor, then the produced ester tends to be more hydrophobic than esters with shorter carbon chain length fatty acids.

Hexadecanoic acid (synonym: palmitic acid) is a fatty acid with a chain length of sixteen carbons (16-C). The presence of a long carbon chain in the fatty acid resulted in high hydrophobic character. The structure of hexadecanoic acid is shown in Figure 2.3.

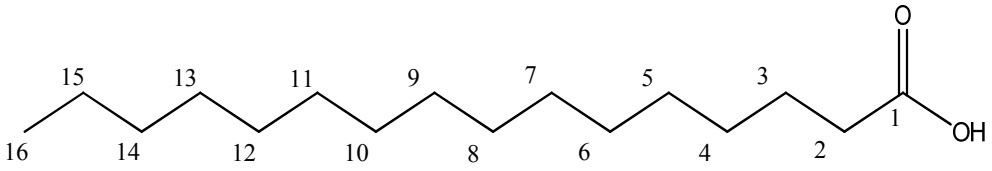


Figure 2.3: Hexadecanoic acid

Tetradecanoic acid (synonym: myristic acid) is a fatty acid with a chain length of fourteen carbons (14-C). Due to a reduced carbon hydrogen (C–H) backbone, it is more hydrophilic compared to the 16-C fatty acid. However, it is more hydrophobic than dodecanoic acid (synonym: lauric acid) (12-C) due to the presence of two extra CH_2 groups in its structure. The structures of tetradecanoic acid and dodecanoic acid are shown in Figure 2.4 and 2.5, respectively.

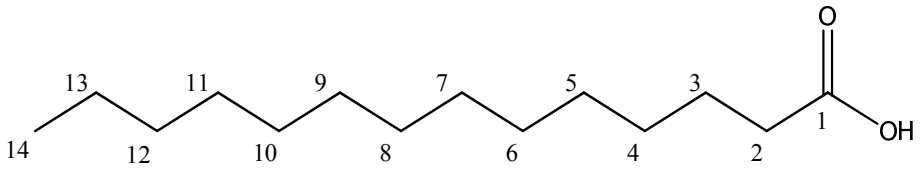


Figure 2.4: Tetradecanoic acid

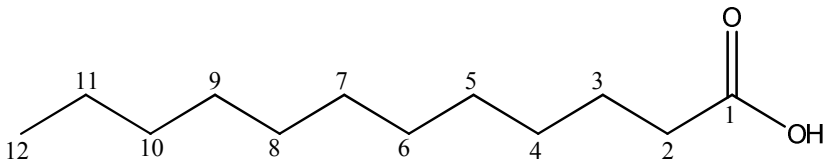


Figure 2.5: Dodecanoic acid

The presence of a carboxyl group (COOH) in fatty acids enables them to be transferred to acyl acceptor group, thus resulting in the formation of new product (e.g. an ester). Long carbon chains of the fatty acids results in the formation of esters with good solubility in hydrophobic environment.

2.3 ESTERIFICATION

Esterification is known to be a process whereby a carboxylic acid or its derivative (as acyl group donor) and an alcohol, e.g. a carbohydrate, (as acyl group acceptor) are transformed into an ester, Figure 2.6:

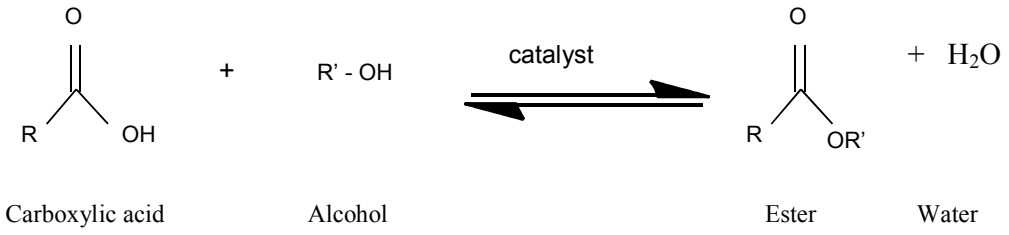


Figure 2.6: Reaction scheme for esterification process. *R* represents a hydrocarbon group.

Carbohydrate esters can be synthesized conventionally *via* chemical routes. This process, however, exhibits several of drawbacks, as it requires high temperature, highly concentrated acid as well as high pressure to drive the reaction. These conditions may lead to side reactions, dehydration of the products as well as product coloration (Sereti *et al.*, 1998). The side products may also possess allergenic and carcinogenic properties (Sarney *et al.*, 2000). To overcome the problem, biological based catalysts have been exploited to drive the esterification (Castillo *et al.*, 2003; Reyes-Duarte *et al.*, 2005; Cauglia & Canepa, 2008). Biological based catalysts provide several advantages to the

esterification process, as the synthesis can be carried out under milder condition (*i.e*: temperature of 60 °C or less, atmospheric pressure of 1 atm) with minimal formation of side products and the process is easier to control.

Direct esterification, or also known as Fischer esterification, was named after the scientist Emil Fischer. Conventionally, this direct esterification uses a highly concentrated acid as catalyst in order to accelerate the esterification process. The Lewis or Brønstedt acid-catalyzed esterification of carboxylic acids with alcohols provides esters as product (Figure 2.6). The reaction (towards product formation), however, is reversible as the produced ester can react with water, thus reforming alcohol and carboxylic acid. This is depicted in Figure 2.6 through the reversed sign. At system equilibrium the forward and reverse reactions are equal leading to steady state conditions. The equilibrium can be shifted to either starting material or product side (*i.e*: prevent the reformation of alcohol and carboxylic acid) by the Le Chatelier's principle. This can be achieved by constant addition of one of the initial substrates (*i.e*: alcohol or carboxylic acid) to force the forward reaction.

Another type of well-known form of esterification is called transesterification, also known as alcoholysis. Transesterification is the displacement of the alcohol component of an ester by another alcohol. It is similar to hydrolysis, except that the water is replaced by an alcohol (Meher *et al.*, 2006). This reaction involves an existing ester (as acyl group donor) and an alcohol (acyl group acceptor) to produce a new ester that exhibits moderate characteristics of both substrates. An example reaction of transesterification is the industrial production of biodiesel. Biodiesel is mostly produced by transesterification reaction of oil with an alcohol in the presence of a catalyst, to yield mono-alkyl esters and glycerol (Akgün & İşcan, 2007). The chemical route of transesterification is shown in Figure 2.7.

Figure 2.7: Chemical route showing the transesterification method utilizing a triglyceride as acyl group donor and methanol as acyl group acceptor.

Like direct esterification, transesterification reaction is also reversible. Increase in the amount of either substrate will force the reaction forward (Le Chatelier's principle). Usually the alcohol is used in excess rather than the starting ester (or glyceride) due to economic advantages and its abundance. The presence of a catalyst can also accelerates the reaction (Meher *et al.*, 2006).

2.4 SURFACTANT

Surface active agents (surfactants) are wetting agents that reduce the surface tension of a liquid, thus allowing easier liquid spreading and able to lower the interfacial tension between two liquids. They have wide variety of uses in daily applications, such as detergents, emulsifiers, stabilizers, as well as conditioning agents. Due to these properties, surfactants are frequently applied in foods, cosmetics, pharmaceutical as well as cleaning industries. The unique interfacial properties of surfactants are explained in molecular level in terms of their structure, in which they contain two characteristically different regions (hydrophobic and hydrophilic regions) in one molecule of surfactant. The presence of these two regions promotes the ability of surfactant to lowers the surface tension of liquid(s) that interact with the surfactant (Kirk *et al.*, 1998). An illustration of a surfactant at a liquid-air interphase is shown in Figure 2.8 (non-ionic surfactant). It is shown that the polar head of surfactant

(hydrophilic region) is attracted to water while the non- polar tail (hydrophobic) of the surfactant immersed in the hydrophobic medium thus lowering the interfacial tension.

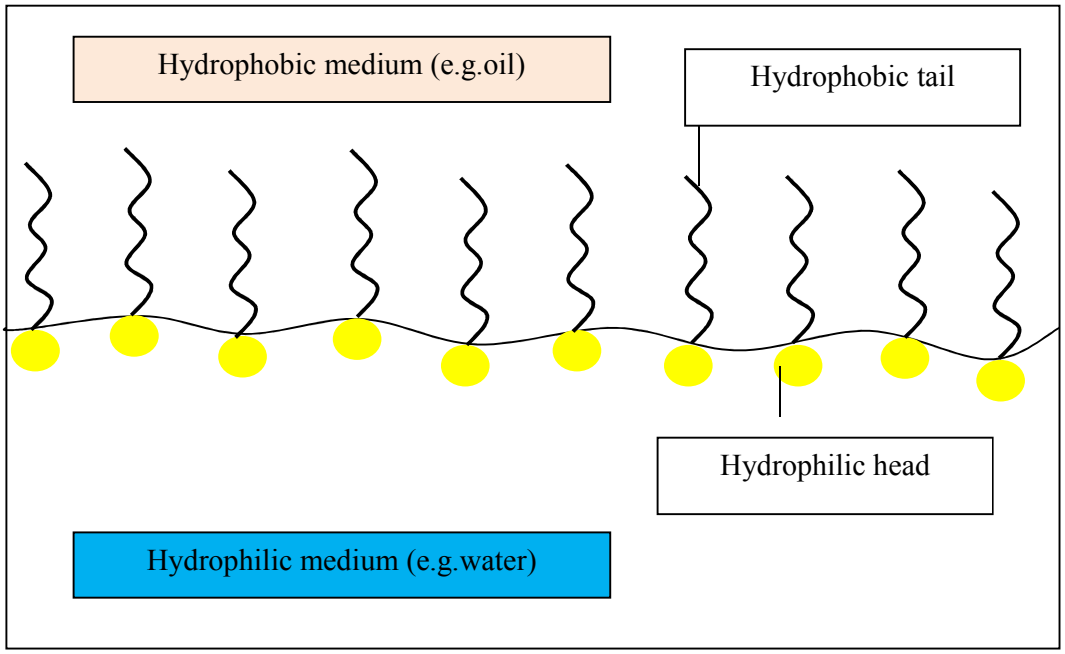


Figure 2.8: Orientation of non- ionic surfactant in the interphase of two liquids of different miscibility characteristics.(Adapted from: http://discovery.kcpc.usyd.edu.au/9.5.5/9.5.5_introsurfactants.html. Accessed on 29th July 2013)

An increase of the surfactant concentration reduces the surface tension until it reaches a minimum at a defined concentration, known as critical micellar concentration (CMC). Higher concentrations of surfactants do not influence the surface tension anymore, as the surfactants begin to associate and organize themselves into complex units, called micelles. The structure of a micelle is shown in Figure 2.9 (Pasquali, 2010).

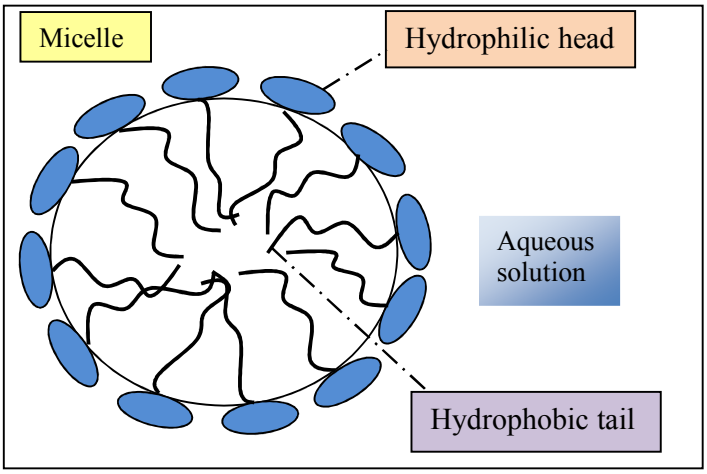


Figure 2.9: Structure of a micelle (Adapted from: http://www.chm.bris.ac.uk/motm/SLS/SLSh.htm. Accessed on 29th July 2013)

Ionic and non-ionic surfactants are types of surfactants that are extensively manufactured and distributed worldwide. The difference in the ionizing capability of a surfactant is determined by the abundance of ions present in the surface active component. If the surfactant comprises of molecules with higher positive charge than negative charge, the surfactant hence is positively charged and known as a cationic surfactant. If the negative charge dominates, the surfactant is called anionic. The difference in net charge of surfactants resulted in different characteristic of the surfactant. The ionic groups are much more hydrophilic than the non-ionic polar groups as the water itself is an ionizing agent.

Negatively charged surfactant (anionic surfactant) are the most widely distributed surfactant (Schmitt, 2001). They are used more than any other surfactants, due to their highly potent detergency and lower cost for their manufacture (Che *et al.*, 2003). They are used in various households as well as in the industrial products and processes. One of the famous anionic surfactant is sodium dodecyl sulphate (SDS), which has been used for nearly 40 years in various fields and is known to be the essential component in shampoo and in toothpaste (Hosseini *et al.*, 2007).

Cationic surfactants, on the other hand, are not used as general purpose detergents as they are not effectively cleaning at pH 7. Due to the natural abundance of negatively charged natural colloids and surfaces, cationic surfactant have high tendency to form a strong adsorption layers on these materials, thus act as efficient stabilizers (Shukla & Tyagi, 2006). In addition, these surfactants are also widely used as fabric softeners as well as antimicrobial agents.

Non-ionic surfactants are the most preferred type of surfactants mainly due to their environmental friendliness. Most of the non-ionic surfactants are low foaming agents and their compatibility with cationic fabric softeners makes them preferable over anionic ones for certain formulations (Schmitt, 2001). They are not subjected to weakening by charged molecules and are, therefore, the best for removing grease stains. Meanwhile, in oil cleaning industries, they tend to be more effective than other surfactants as they have a wide range of hydrophilic-lipophilic balance (HLB) values, from 1 to 20. This HLB value is used as an indicator for surfactant miscibility with water and oil. Non-ionic surfactants with low HLB value (< 10) are known to be miscible with oil, While higher HLB values (10 < HLB < 20) indicate water friendly surfactants. A simplified correlation of properties and HLB for non-ionic surfactants is shown in Figure 2.10. Carbohydrate fatty acid esters are one of the well- known non-ionic surfactants. Their appeal and demand in the market increases steadily due to their versatile characteristics as well as their biodegradability in nature.

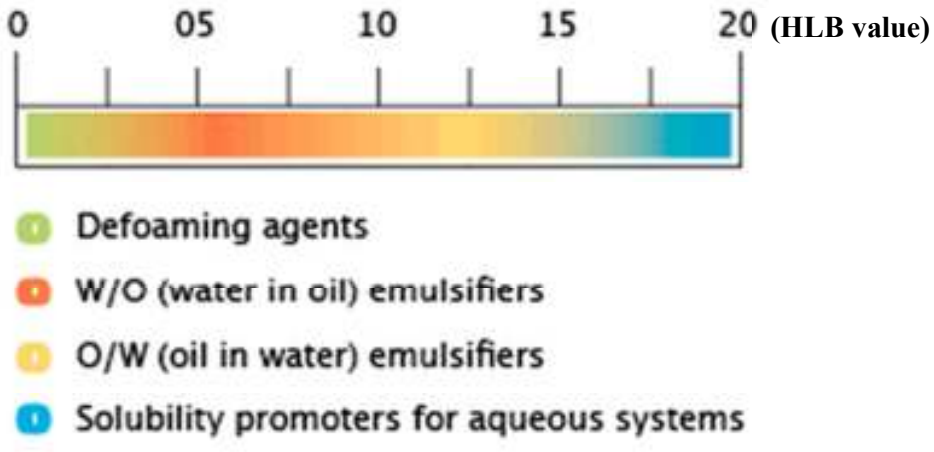


Figure 2.10: Properties of non-ionic surfactants according to their HLB value (Source: http://www.britznetworks.com/en/products/polymer-additives/emulsifiers. Accessed on 22nd February 2013).

2.5 CARBOHYDRATE ESTER

Carbohydrate esters are among those surfactants that belong into the category of biodegradable non-ionic surfactant. They are widely used as surfactants due to their high capability as emulsifying agents for oil-in-water and water-in-oil mixtures, as they possess a wide range of HLB values. The value of HLB depends upon the kind of acyl donor and acyl acceptor applied in the system. The stronger the effect of acyl acceptor compared to acyl donor group in a carbohydrate ester the higher its HLB value and *vice versa*. Their main characteristic of being readily biodegradable promotes them as environmental friendly surfactant. Because of that, they have become a favourite surfactant for daily and mass usage, especially for industrial detergents and as food emulsifiers (Coulon *et al.*, 1995; Holmberg, 2001; Šabeder *et al.*, 2006; Yoo *et al.*, 2007).

Carbohydrate esters comprise of two different molecular regions with extremely different physical and chemical properties. Varying degrees of hydrophilicity and hydrophobicity for carbohydrate esters depend on the carbohydrate and fatty acid the ester is made of. Utilization of disaccharides for the carbohydrate produces surfactants

with higher polarity. This is in contrast to the utilization of longer chained fatty acids as the acyl donor; the presence of larger hydrocarbon backbones of the fatty acid increases the hydrophobicity of the produced carbohydrate ester. Table 2.1 summarises commercial carbohydrate esters available in industry.

Table 2.1 : Some of available commercial carbohydrate esters in industries (H. Razafindralambo *et al.*, 2012).

Carbohydrate ester	**Selected Suppliers**	**Field of Applications**	**Production Capacity, world [t/a][a]**
Sorbitan esters	Akzo Nobel, Cognis, Dai-Ichi Kogyo Seiyaku, Kao, Riken Vitamin, SEPPIC	Pharmaceuticals, personal care, food, fiber, agrochemicals, coatings, explosives	20,000
Sucrose esters	Cognis, Croda, Dai-Ichi, Kogyo Seiyaku, Evonik/Goldschmidt, Mitsubishi-Kagaku, Sisterna, Jiangsu Weixi	Food, personal care, pharmaceuticals	<10,000
Alkyl polyglucosides	Akzo Nobel, BASF, China Research Institute of Daily Chemical Industry, Cognis, Dai-Ichi Kogyo Seiyaku, Kao, LG, SEPPIC	Personal care, detergents, agrochemicals	85,000
Others: Methylglucoside esters; Anionic alkyl polyglucoside derivatives	Lubrizol/Noveon Cognis, Cesalpina	Personal care, pharmaceuticals, personal care	<10,000

2.6 LIPASE

Lipases (*EC 3.1.1.3*) have been extensively exploited for enzyme catalyzed esterification. *Candida antarctica* lipase (CAL B) is known to have the capability to drive the reaction (Arsan & Parkin, 2000; Al-Zuhair, 2005; Yoo *et al.*, 2007; Zoumpanioti *et al.*, 2008). This enzyme belongs to hydrolase family with a conserved catalytic group of Ser, His, and Asp/Glu (Uppenberg *et al.*, 1995). In water-rich environment, lipases are known for their hydrolytic reaction and they tend to hydrolyze triacylglyceride to produce glycerol and three fatty acids (Figure 2.11). However, the role is reversed when the lipases are in micro-aqueous environment. Here, they tend to catalyze the formation of ester bonds in presence of both acyl donor and acyl acceptor groups.

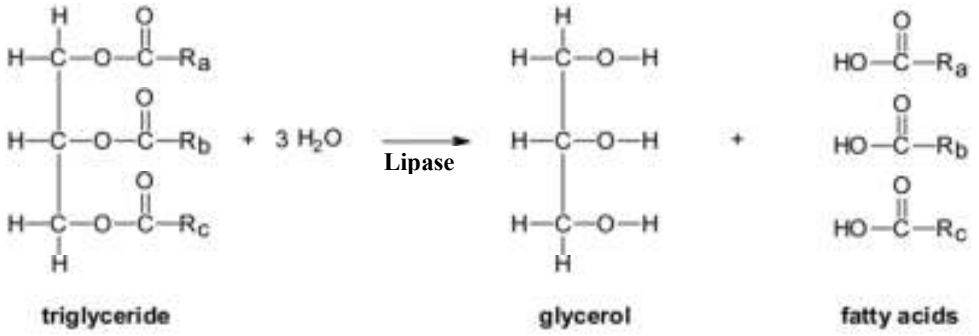


Figure 2.11: Hydrolysis reaction produces glycerol and fatty acids from fats and oils. In the fatty acids, R_a, R_b, and R_c, represent groups of carbon and hydrogen atoms in which the carbon atoms are attached to each other in an non-branched chain.

CAL B belongs to the α/β-hydrolase-fold superfamily (Ollis *et al.*, 1992), which contains enzymes that evolved from a common ancestor to catalyze various hydrolysis reactions of esters, thioesters, peptides, or cleavage of carbon bonds in hydroxynitriles (Holmquist, 2000). CAL B is a built up of 317 amino acids and has a molecular weight of 33 kDa. (Magnusson, 2005). The structure of enzyme CAL B is shown in Figure 2.12.

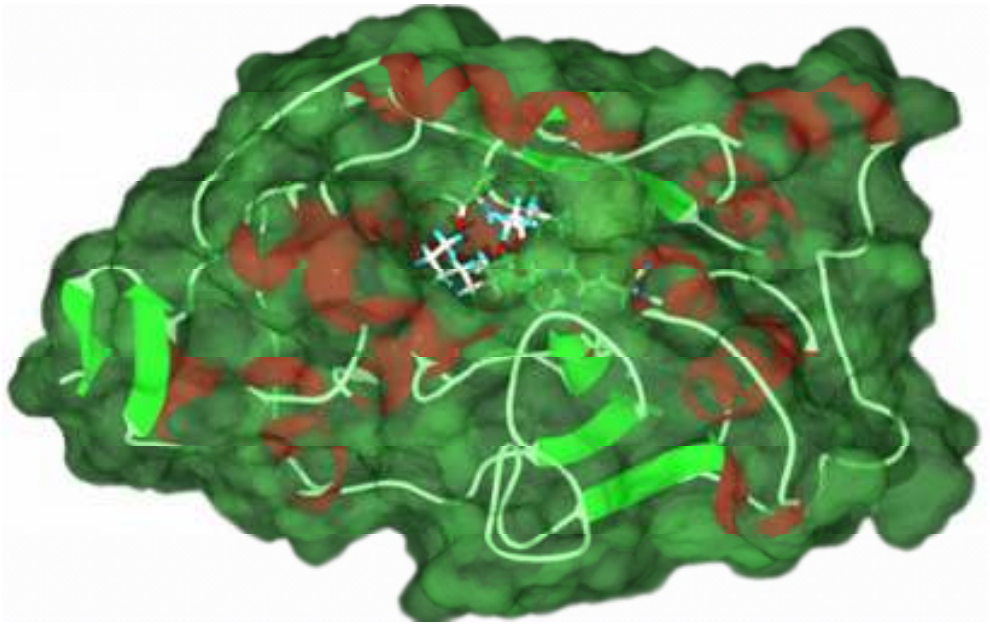


Figure 2.12: Structure of *Candida antarctica* lipase B that belongs to the α/β - hydrolase- fold super family. The α-helices are shown in red, the β-sheets in pale green, and the enzyme surface is in dark green (Magnusson, 2005).

Organic solvents were utilized as media for enzyme (CAL B) catalyzed esterifications. CAL B in organic solvent is to retain a catalytic efficiency similar to those in water (Secundo & Carrea, 2002; Halling, 2004; Trodler & Pleiss, 2008). The CAL B in organic solvents is more thermostable and possesses high stereo- and regio-selectivity in esterifications (Sereti *et al.*, 1998).

Beside the catalytic efficiency of the enzyme, other limitations need to be given consideration in order to improve the catalytic efficiency in solvent based reaction. High substrate concentrations, restricted protein flexibility, diffusional limitation and low stabilization of substrate-enzyme complex have been found to be among the causes for reduced efficiency in solvent based reaction (Klibanov, 1997; Dodson & Verma, 2006). The flexibility of CAL B generally decreases with increasing solvent partition coefficient (Log *P*) as well as the decreases with the solvent dielectric constant (Trodler & Pleiss, 2008). The structure of CAL B however was found to be independent of the

environment and no alteration in its conformation in different solvents was found (Martinelle & Hult, 1995; Uppenberg *et al.*, 1995).

Low water content in the organic solvents was one of the causes for reduced efficiency in enzyme catalyzed reactions in organic solvents. The decrease in solvent accessible surface on the enzyme, especially of the hydrophilic surface, resulted in low reaction efficiency. Two types of water that interact with enzyme are differentiated in organic solvents, *viz.* the 'inside' water that is bound to the interior of the protein thus forming hydrogen bonds, which affect conformation of the active site of enzyme, and 'contract' water that is weakly bonded to the surface of the enzyme and exchanges rapidly with non-enzyme associated water in the solvent (Nakasako, 2004). Mobility of water is restricted in organic solvent compared in an aqueous medium. This reduces the flexibility of the enzyme in the organic solvents due to lack of interaction of water from the organic solvent with the enzyme bound water (Trodler & Pleiss, 2008).

Lipase is known to exhibit high selectivity in esterifications. The attachment of the acyl group from the acid to a carbohydrate (monossacharide) favours the carbon 6 (C-6) (Ferrer *et al.*, 2005; Cauglia & Canepa, 2008), thus resulting in the formation of carbohydrate monoester.

2.7 BIOSYNTHESIS OF CARBOHYDRATE ESTERS

Carbohydrate esters are produced by inter-esterification reaction between a fatty acid (as acyl donor) and a carbohydrate (as acyl acceptor). The process can be carried out by either chemical or biological routes. The chemical synthesis of sugar ester requires carbohydrate pre-treatment, dehydration of the carbohydrate (*i.e*: dehydration of sucrose in presence of highly concentrated sodium dihydrogen phosphate) at high temperature (150 to 200 °C), and subsequent transesterification with fatty acid catalyzed by base (disodium carbonate) at high temperature (200 to 250 °C) (Behler *et al.*, 2001).

A milder way of synthesizing the sugar esters was extensively studied in order to satisfy the world demand for a more environmental-friendly manufacturing process. Enzymatic catalyzed sugar ester production was discovered and extensively used in the process for sugar ester production. However, there were limitations in terms of conducting the process itself. Lipase, a hydrolase, was found to catalyze such system reactions in absence of water. It is well known that carbohydrates are strongly polar substrates and, hence, solubility of the substrate becomes one of major limiting factor in the reaction since the medium used is not aqueous (Sereti *et al.*, 1998; Flores *et al.*, 2002). Apart from that, synthesis of sugar ester through biological routes is reversible (Reyes-Duarte *et al.*, 2005). In presence of water the lipase reacts in its conventional way thus hydrolyzing the fatty acid-lipase complex intermediate. This results in low reaction efficiency, as shown in a schematic reaction diagram in Figure 2.13.

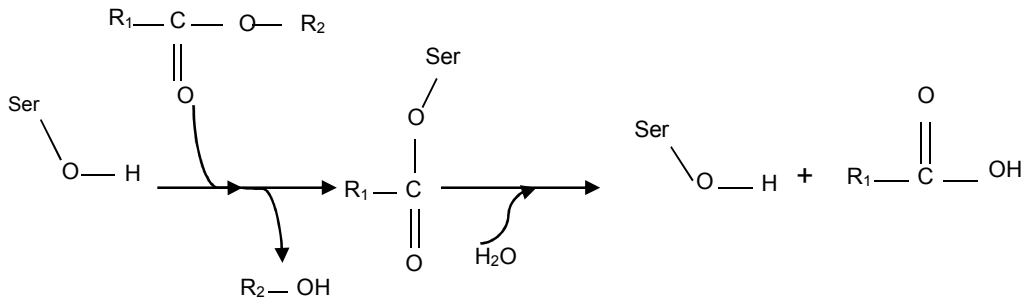


Figure 2.13: Hydrolysis of acyl donor- lipase complex

There are several methods for water removal in a reaction. The use of molecular sieves is one of the preferred ways to remove water generated during an esterification. However, increase in the amount of molecular sieves with the amount of organic solvent medium may resulted in mass transfer limitation due to difficulty in mixing (Yan *et al.*, 1999; Zhang *et al.*, 2003). Reaction in absence of organic solvent as the reaction medium was used in the esterification process (Xu *et al.*, 2003). Azeotropic distillation, gas sparging as well as pervaporation were also studied and applied to systems in order to remove generated water (Kwon *et al.*, 1995; Won & Lee, 2001). Evaporation of water under reduced pressure during the reaction, however, resulted in limited product formation (Mukesh *et al.*, 1993; Ducret *et al.*, 1998).

One of the indicators for solvent polarity is based on its octanol-water partition coefficient (Log *P*). Substrate (acyl donor acceptor group) solvation limitation in the reaction media due to polarity constraint has been improved through several modifications, one of which is the blending of two or more solvents (Degn & Zimmermann, 2001; Fu & Vasudevan, 2010; Gumel *et al.*, 2011; Valepyn *et al.*, 2011). Blending of a solvent of high polarity *viz.* dimethylsulfoxide (DMSO) (Log *P* = -1.35) with one of lower polarity, e.g. *tert.* butanol (Log *P* = 0.35) at 1:1 (v/v) ratio, resulted in a reaction mixture exhibiting an intermediate polarity value, e.g. -0.62. The system was

able to dissolve higher substrate contents compared both solvents in pure form (Gumel *et al.*, 2011).

2.8 STATISTICAL EXPERIMENTAL DESIGN

Conventionally, optimization experiments are conducted by applying a one factor variation only. For instance, in a reaction system where involving more than one factor, e.g. A and B, the experimenter chooses either A or B as the first factor to be investigated thus manipulating the said parameter to find its effect towards the reaction. The same procedure is applied to the second factor. This procedure, however, has a weakness, because the experimenter cannot find out whether the factors A and B interact with each other. A statistical design of experiments allows the experimenter to plan experiments, which the data is analyzed by statistical methods and enable conclusions on single factors as well as on their interactions. The method is more crucial when the data are subjected to experimental errors. In this case, the data can only be analyzed in an objective manner through statistical analysis.

The quality of data obtained from any statistical experimental design relies on several principles. A basic principle of any experiment including those in a one factor investigation is a randomization of experiments. This is important as a statistical analysis requires the observation data to be independently distributed random variables. Replicating experimental runs, which is another basic requirement in obtaining reliable data from any experimental design, is also crucial when applying a statistical experimental design. Replication provides advantages as the experimenter can estimate the experimental error as well as the experimenter has the capability to evaluate whether an effect is statistically validated or no (Montgomery, 2008).

Factorial statistical experimental design is one of available methods to conduct experiments involving a numbers of variables. Factorial experimental design allows investigation of several factors involved in an experiment in complete replication at all levels of the factors. General factorial experimental design consists of 2 levels (high and low) for every variable tested. Factorial statistical experimental design allocates information on the effect of specific factor to the experiment. This can be seen from the main effect plot in the analysis of a factorial experimental design. An example for the effect of two level temperatures on an experimental response in main effect plot is shown in Figure 2.14.

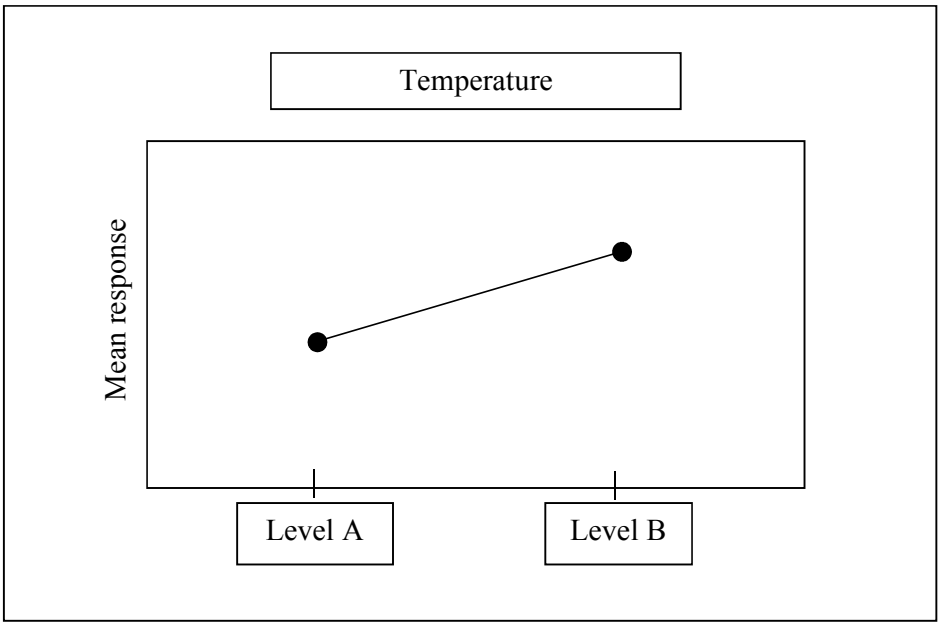


Figure 2.14: Main effect plot for two-level factorial experimental design

There are two ways in conducting factorial experimental design, *viz*. full factorial experimental design and fractional factorial design. Full factorial experimental design requires the experimenter to conduct all factor combinations for an experiment. For instance, if there are three factors involved in the experiment, a total of 8 runs is required excluding replications. A total number of 24 runs are required based on 2^3 if three replicates of the same run are included in the design. By including three replicates of one centre point in order to find whether there is a curvature present in between the high and low level of every factor, the sum of all runs for the experiment increases to 11 or 27, depending on requirement of replicates. The number of required experiments is very large. In order to speed up the process an experimenter has the option whether to run a fractional factorial experimental design instead.

A fractional factorial experimental design is particularly beneficial if high numbers of factors are involved in the investigation. For example, in a full factorial experimental design of a five factors experiment requires 2^5 basic experiments, which is equal to 32 runs. This can be reduced to either eight (1/4 fraction) or sixteen (1/2 fraction) runs depending on the fraction design that the experimenter uses. Fractional factorial design is among the most widely used type of design for product and process optimization. Several considerations however need to be taken into account when analysing the fractional factorial design. Some of the effects in this design are confounded with each other. These effects cannot be analysed separately thus the specific fraction must be carefully chosen so that viable results are generated (Montgomery, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Enzyme lipase acrylic resin of *Candida antarctica* (recombinant expressed in *Aspergillus niger*) (Novozym 435) *(E.C 3.1.1.3)* was purchased from Sigma Aldrich. Methyl α-*D*-glucopyranoside from Merck was chosen as acyl acceptor. Hexadecanoic acid, tetradecanoic acid and dodecanoic acid from Merck were used as acyl donors. Sulphuric acid, chloroform, *n*-hexadecane and potassium dichromate were also purchased from Merck. Laboratory grade *n*-butanol, acetic acid, ethanol 99.9 %, methanol, *n*-hexane were purchased from Systerm. 4Å molecular sieves, *para* nitrophenyl palmitate (*p*NPP), deuterated chlorofom with Trimethylsilane (TMS) as internal standard and Karl-Fisher titrant Hydranal Coulomat AG were purchased from Sigma Aldrich. Analytical grade *tert.* butanol as reaction medium was purchased from Ajax Finechem. Laboratory grade Sodium Chloride (NaCl) and Sodium Hydroxide (NaOH), were purchased from Systerm. NMR tubes for 300 MHz spectrometers were purchased from Duran and Thin Layer Chromatography (TLC) silica gel 60 F_{254} plates were purchased from Merck.

3.2 General flow of the study

The study consisted of six stages of experimental works. The workflow is shown in Figure 3.1.

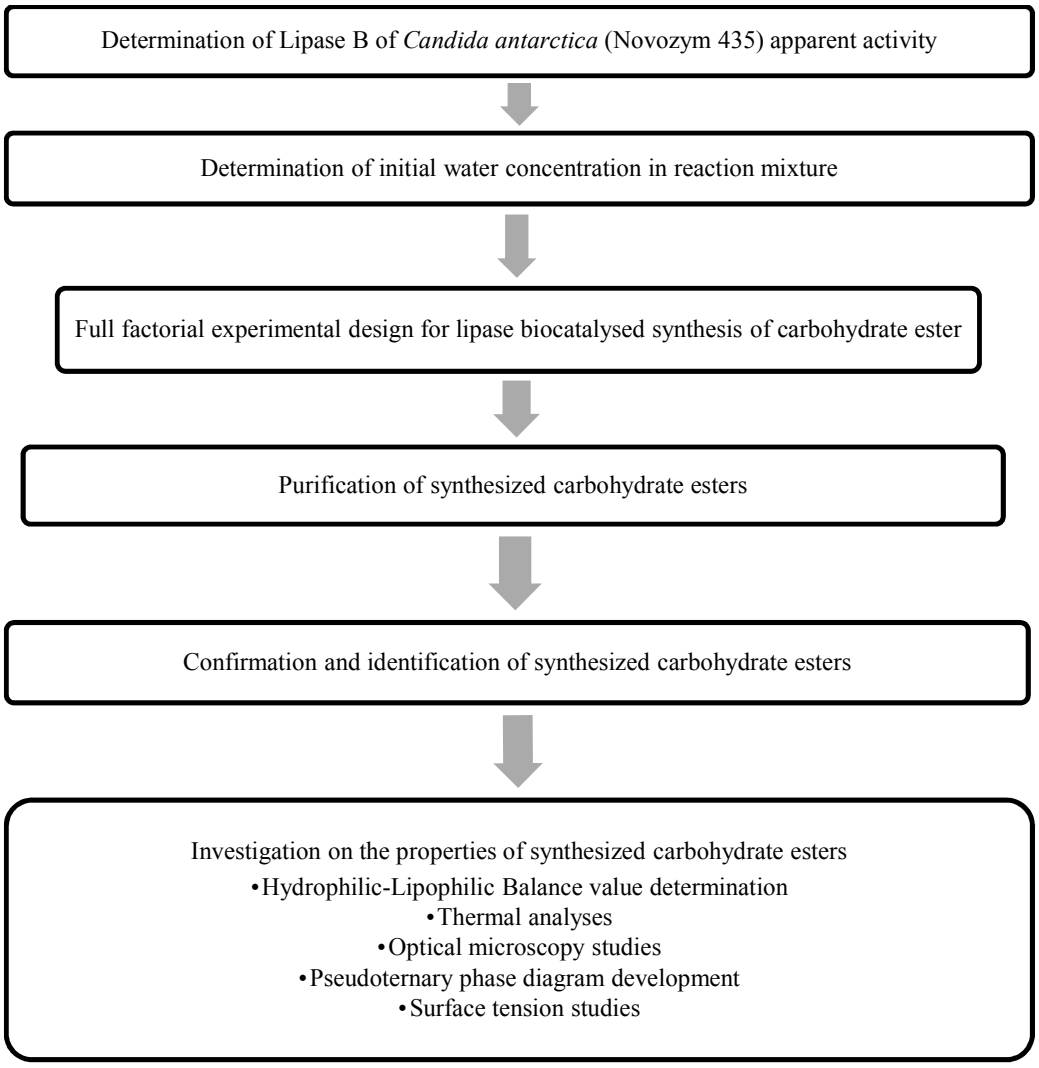


Figure 3.1: General workflow of the research

3.3 Determination of the Lipase B apparent activity

Apparent activity of lipase enzyme used in the reaction was determined through ester hydrolysis reaction. 10 mM *para*- nitrophenyl palmitate (*p*NPP) in *n*-hexane was used as substrate as described by Pencreac'h and Baratti (1996) with some modification *viz*. *n*–hexane was used as the dissolving medium for *p*NPP. The hydrolysis reaction was carried out in a two phase solution where immobilized lipase will partition between hexane and 0.1 M $NaOH_{(aq)}$ solution interphase. Hydrolysis of *p*NPP releases *p*-nitrophenol as one of the product and this was extracted into the 0.1 M $NaOH_{(aq)}$ thus resulting in yellow coloration in the alkaline aqueous phase. Method for *p*-nitrophenol molar absorption coefficient determination is shown in Figure 3.2.

Molar extinction coefficient (ε) of *p*- nitrophenol in *n*-hexane was determined in order to calculate lipase apparent activity *via* Beer –Lambert relationship. The equation used for calculating *p*- nitrophenol molar absorption coefficient is shown in Equation 3.1.

$$A = \varepsilon \times l \times C$$

$$\varepsilon = \frac{A}{l \times C} \times dilution\ factor \qquad \text{(Equation 3.1)}$$

where,

A = Absorbance

ε = Molar extinction coefficient (L mol^{-1} cm^{-1})

C = Concentration of *p*-nitrophenol (mol L^{-1})

l = Cuvette path length (1 cm)

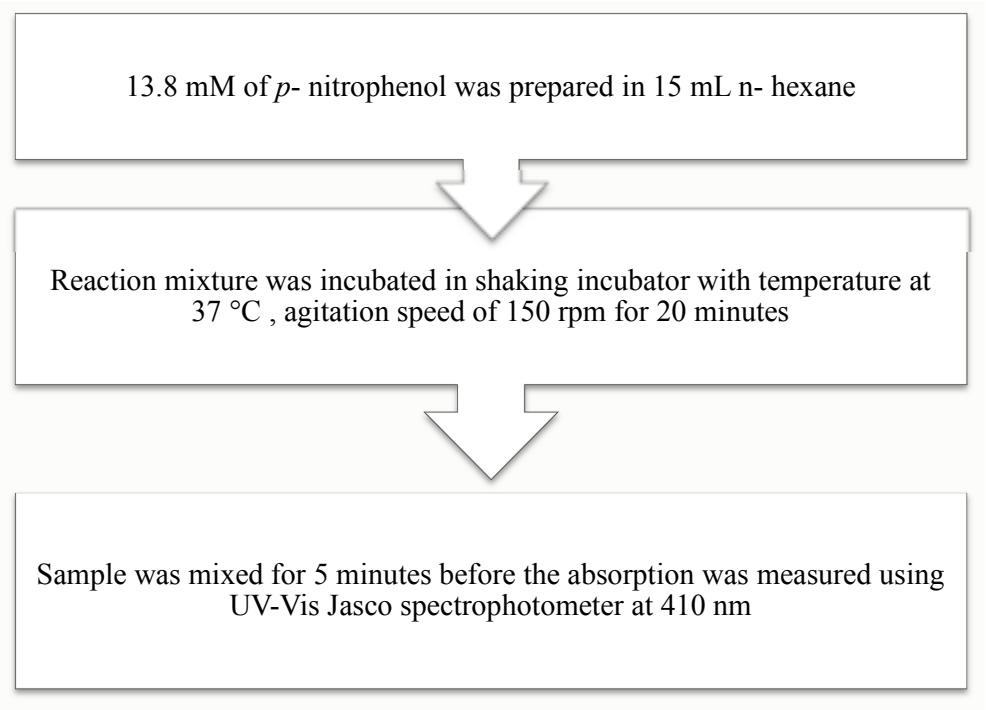


Figure 3.2: Flow chart for *p*-nitrophenol molar absorption coefficient determination.

Apparent activity of lipase was then determined using time course measurement in a temperature controlled UV-Vis spectrophotometer at wavelength 410 nm. The optimum temperature for lipase activity was within the range of 40°C until 50°C (Krieger *et al.*, 2004; Yu *et al.*, 2013). Hence, the temperatures used for determination of lipase apparent activity were in the range of 35 °C until 57 °C (*i.e*: 35°C, 43°C, 50°C and 57°C). Method for apparent lipase activity determination is shown in Figure 3.3:

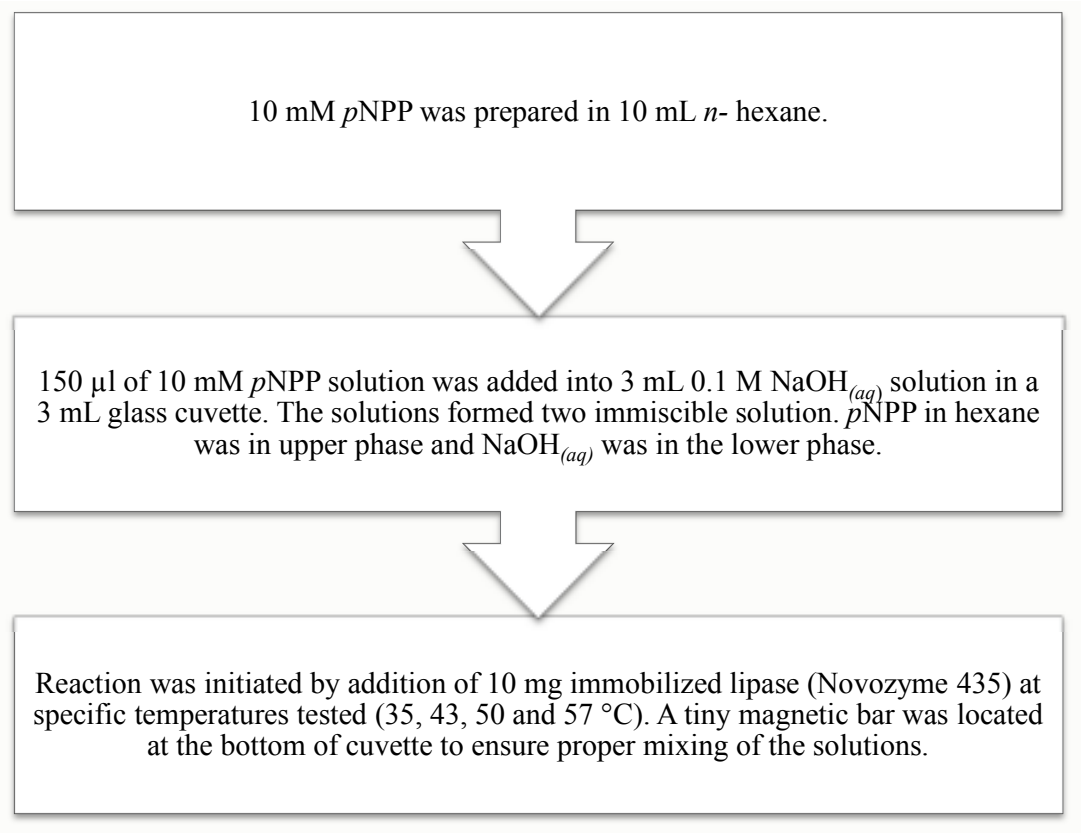


Figure 3.3: Flow chart for immobilized lipase (Novozym 435) apparent activity measurement.

Lipase apparent activity was calculated using Equation (3.2):

$$\text{Activity}_{(app)} = \frac{\Delta\,\text{Abs}_{410\,nm} \times (V_S)}{V_{ls} \times (l) \times (\varepsilon_{410})} \qquad \text{(Equation 3.2)}$$

where,

Δ Abs $_{410\,nm}$: Different in absorbance at 410 nm at known time interval (min^{-1})

V_s : Total assay volume (mL)

V_{ls} : Volume of substrate (mL)

l : path length of cuvette (1 cm)

$\mathcal{E}_{410}$: Extinction coefficient of *p*- nitrophenol in 0.1 M NaOH at 410 nm

3.4 Determination of initial water concentration

Initial water content of reaction solvent was determined using Metrohm 831 Karl- Fischer coulometer. The method quantify amount of water dissolved in the sample through reaction of iodine with water. Hence, the amount of iodine generated directly into the sample reflected the amount of water present in the reaction mixture. Amount of water present in the system was reported as weight of water content per sample weight (ppm).

1.0 mL of sample was pipetted out from the reaction mixture and was put onto the weighing balance model Mettler- Toledo AL 204 as shown in Figure 3.4 (a). The balance was tared to zero in the presence of the sample. Sample was then injected into the vessel containing Coulomat® reagent through the septum as shown in Figure 3.4 (b). The remaining weight was measured again without re-zeroing the weighing balance. Value indicated on the balance was keyed into the Karl-Fischer titrator processor using the external keyboard (Figure 3.4 (c)). Amount of water presence in the respective amount of sample injected into the titrator was then displayed on the screen (Figure 3.4 (d)) in ppm.

Figure 3.4: Set up for water content determination using Karl- Fischer Metrohm 831 titrator.

3.5 Full factorial experimental design

Experimental design to study the effects of selected process parameters towards the efficiency of carbohydrate ester biosynthesis was performed using full factorial experimental design in MINITAB® (15) software. Full factorial experimental design was applied to the study of the effects of two or more factors tested in the experiment. This allows the experimenter to know which main effect exerts significant influence on the system (Montgomery, 2001). In this study, six (6) factors were tested in the design *viz.* ratio of acyl donor to acyl acceptor (mol: mol), enzyme loading (g), 4Å molecular sieve amount (g), agitation speed (rpm), reaction temperature (°C) and incubation time (hour). Table 3.1 showed a two (2) level full factorial design (coded value with center point doing with real value) applied into the system. The total runs for esterification is shown in Table 4.3 in Chapter 4.

Table 3.1: Coded and real value variables used in the full factorial screening experiment.

Code	Mol ratio (acyl donor : acyl acceptor) (mmol)	Reaction temp. (°C)	Quantity enzyme (g/ g acyl acceptor)	Quantity 4Å molecular sieve (g/ 8.1 g solvent)	Agitation speed (rpm)	Incubation time (hr)
1	3 : 1	50	2.0	1.00	200	24
0	2 : 1	40	1.0	0.50	100	16
-1	1 : 1	30	0.5	0.25	50	8

3.5 General carbohydrate ester bio-synthesis conditions for full factorial experimental design

Bio-synthesis of carbohydrate ester was performed in *tert.* butanol as the reaction solvent. The water content of the solvent was lowered prior to usage at 30 ± 3 °C overnight using 4Å molecular sieves in a tightly sealed screw cap bottle. A reaction mixture contained pre-determined amounts of acyl donor, acyl acceptor, fresh 4Å molecular sieves and *tert.* butanol. Reaction was initiated with addition of immobilized lipase from *Candida antartica* B (Novozyme 435). A control reaction (without lipase) was also prepared in parallel to the reaction mixture in order to get the actual amount of dissolved methyl α-*D*-glucopyranoside in the *tert.* butanol solvent at a particular incubation time and temperature. The tightly sealed vials containing the reaction mixture were then agitated in an incubator shaker at a pre-determined shaking speed (rpm), incubation time (hour) and temperature (°C).

The experimental parameters for bio-synthesis of carbohydrate ester were previously shown in Table 3.1.

3.6 Product monitoring and assay

Carbohydrate ester was quantified indirectly using residual carbohydrate as response parameter. 0.5 mL of reaction mixture was pipetted out and filtered using PTFE filter in order to remove traces of immobilized enzyme and the non- dissolved carbohydrate. 0.5 mL of *n*-butanol was then added into the filtrate and mixed. Extraction of unused carbohydrate from the solution was done *via* salt water -alcohol extraction. 1 mL of 20 % (w/v) NaCl solution was added into the 1 mL of *n*-butanol – *tert.* butanol reaction mixture and was vortexed in order to mix the solutions. The solution was then poured into a separating funnel and allowed to stand until two separate layers formed (the lower phase: salt water and unused carbohydrate, upper phase: *n* butanol – *tert.* butanol- fatty acids- carbohydrate ester). The lower phase was then recovered and subjected to evaporation under reduced pressure at 70 °C to remove traces of *tert.* butanol that may dissolve out from the upper phase into the salt water phase. Equal volume of distilled water with reference to the initial salt water was pipetted into the recovered solid and then mixed. Quantitative analysis of the residual carbohydrate was done using phenol sulphuric acid method and measured at 490 nm using a UV-Vis Jasco model 630 spectrophotometer. Efficiency of esterification was calculated using Equation (3.3).

$$\left[1 - \frac{(\text{residual methyl } \alpha - D - \text{glucopyranoside})_{\text{reaction}}}{(\text{methyl } \alpha - D - \text{glucopyranoside})_{\text{control}}}\right] \times 100$$

(Equation 3.3)

3.7 Purification of carbohydrate ester

Reaction mixture was filtered using PTFE filter to remove the immobilized enzyme lipase, 4Å molecular sieve as well as the non- dissolved methyl α–*D*-glucopyranoside. The filtrate was then subjected to evaporation under reduced pressure at 70 °C to remove the solvent *tert.* butanol. Hexane was added to the remaining solids and the mixture was centrifuged to remove the residual acyl donor. The procedure was repeated three times. Subsequent steps for product separation depend on the length of carbon chain of acyl donor used.

a) For acyl donor >12 C: Water: chloroform (1:1 v/v) was added to the solid and vigorously mixed. The solution was let stand until two separate liquid layers were formed. The upper layer which consists of water and residual carbohydrate was then pipetted out and carbohydrate ester was obtained in the interphase between water and chloroform.
b) For acyl donor (12-C): Water: chloroform (1:1 v/v) was added to the solid and vigorously mixed. The solution was then let stand until two separate liquid layers were formed. The upper layer which consists of water and residual carbohydrate was then pipetted out and carbohydrate ester was obtained in the lower chloroform phase.

3.8 Product confirmation and identification

3.8.1 Thin Layer Chromatography

Initial evaluation on the carbohydrate ester formed was done using thin layer chromatography (TLC) with developing reagent of chloroform: methanol: acetic acid: water (70: 20: 8: 2 vol/vol) and visible reagent of sulphuric acid: ethanol: water (2: 90: 8 vol/vol).

Spotting of reaction sample onto TLC plate at mark A was done using Pasteur pipette as the spotter (Figure 3.5 (A)). Spotted TLC plate was then inserted into Mc Cartney bottle containing developing reagent. Once the solvent's front reached mark B (Figure 3.5 (B)), the plate was then taken out and let dry before being dipped into Mc Cartney bottle containing visible reagent. TLC plate was then dried by applying heat until spots at specific distance appeared on the plate. R_f calculation was done by dividing distance from (A) to the product's spot with total distance from (A) to (B). Marks (A) and (B) are shown in Figure 3.5 and TLC with products is shown in Chapter 4 (Figure 4.3).

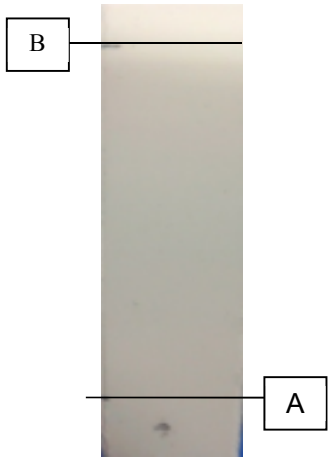


Figure 3.5: A new thin layer chromatography (TLC) plate with (A) initial and (B) terminal marking.

3.8.2 Nuclear Magnetic Resonance (NMR) spectroscopy

Further authentication of the product was done *via* NMR spectroscopy at 270 MHz. Purified product was dried overnight in vacuum oven at 45 ^{o}C in order to remove excess water within the product. Small amount of product (carbohydrate ester) was dissolved into deuterated chloroform (with trimethylsilane as internal standard). The solution was filtered in order to prevent any precipitation that can interfere with the NMR before being pipetted into the 300 MHz NMR tube. A total of 1 mL filtered sample solution was pipetted into the NMR tube.

3.9 Hydrophilic-Lipophilic Balance (HLB) value calculation

HLB values for all carbohydrate esters synthesized were calculated in order to find their degree of water loving properties. The degree of carbohydrate ester solubility in water and in oil was explained previously in Chapter 2. The value of the HLB for every carbohydrate ester synthesized was calculated using Equation (3.4):

$$\text{HLB value} = 20 \times \frac{\text{Molecular weight } (M_w) \text{of hydrophilic group}}{\text{Total molecular weight of the molecule}} \qquad \text{(Equation 3.4)}$$

3.10 Properties of synthesized carbohydrate ester

3.10.1 Thermal analysis of methyl α-*D*-glucopyranoside ester

The effects of temperature on the behaviour of synthesized methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (12-C carbohydrate ester), methyl 6-*O*-tetradecanoyl-

α-*D*-glucopyranoside (14-C carbohydrate ester) and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside (16-C carbohydrate ester) were studied using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The study was performed using a Perkin-Elmer differential scanning calorimeter of model DSC 6 (Perkin-Elmer Inc., Wellesley, MA, USA). Measurements were performed under a nitrogen flow rate of 50 mL min^{-1}. Solids of purified carbohydrate esters were weighed and samples in the range of 2-3 mg were placed on a pan inside the DSC alongside an empty reference pan. The thermal effect towards the change in behaviour of carbohydrate esters was investigated over a heating temperature range of from 0 - 130 ^{o}C in a heating and cooling cycle applying a rate of 10 ^{o}C min^{-1}. The heating and cooling was conducted in two cycles. TGA analysis was performed on a Perkin-Elmer TGA 4000 instrument. The sample was heated from 30 °C to 900 °C at a rate of 10 °C min^{-1} under a nitrogen flow rate of 20 mL min^{-1}.

3.10.2 Optical Polarized Microscopy (OPM)

3.10.2.1 Thermotrophic study

Visualization of morphology changes of carbohydrate esters with temperature, referring to liquid crystal properties were investigated using OPM (Figure 3.6) on a Mettler Toledo FP82HT hot stage and viewed with an Olympus BX51 microscope fitted with crossed polarizing filters. The microscope was connected to a camera for image capture and analyzed using the AnalySIS Imager software. Samples of carbohydrate ester were transferred onto a microscope glass slide. The solids were equally distributed by pressing them before applying the cover slip onto the sample. The slide was then mounted on a temperature controlled microscopic slide holder (Mettler Toledo FP82HT hot stage) and subsequently placed on the microscope's stage. The temperature range used for analysis was 30 – 105 °C. Two cycles of heating and cooling was performed

before images of textures were recorded. Presence of liquid crystals in the samples can be verified by appearance of textures under crossed polarizers. An example is shown on the monitor in Figure 3.6 (a). Absence of liquid crystals in samples on the other hand showed a black texture, hence no image.

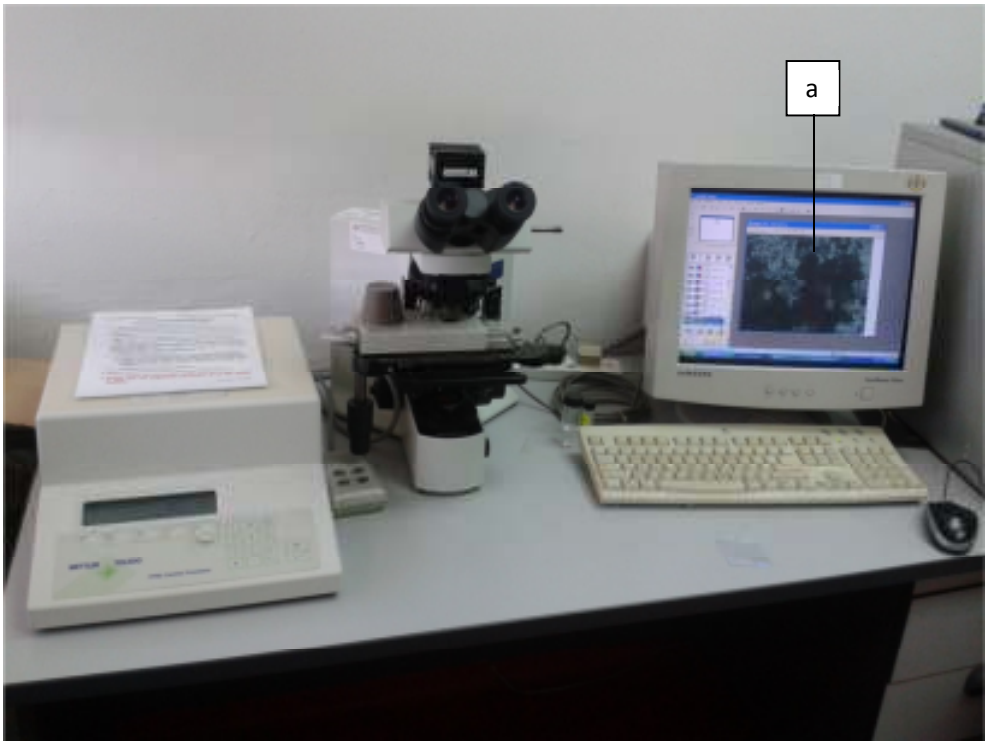


Figure 3.6: Optical polarized microscope set up for thermotrophic analysis on carbohydrate esters. (a) Represents liquid crystal structures in the sample.

3.10.3 Pseudoternary phase diagram development

Development of phase diagram will provide information on emulsion formation (one phase homogenous solution) and separation of the formed emulsion (heterogenous solution) at specific ratio of all different components within the system. In this study, *n*-hexadecane was used as model oil together with *n*-butanol as the co-solvent.

The capability of emulsion to form using synthesized carbohydrate esters were investigated using methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside, and methyl 6-*O*-hexadecanoyl-α-*D*-

glucopyranoside. The experiment was conducted in thermostatic water bath at elevated temperature of 60 °C. The solution was titrated with water (0.005 mL per titration) and mixed vigorously using a vortex mixer at every interval until the solution became turbid. The appearance of turbid solution was used as an indication of phase separation. The weight ratio between oil and co-solvent was kept constant throughout the experiment at 1:1 (w/w) (Khiew *et al*., 2004). The samples that remained transparent and homogenous after vigorous vortex were considered within the monophasic area in phase diagram (Garti *et al.,* 2000).

3.10.4 Surface tension studies

A surface tension study was performed on a KSV Sigma tensiometer (702 series). The study was only performed with methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside at an elevated temperature of 55 °C. It was found that carbohydrate esters with higher fatty acid chain length showed poor solubility in water. This limits further investigations. The study is important to investigate, whether the mentioned carbohydrate ester possesses surfactant properties, *i.e* ability to reduce the surface tension at the air-water interphase.

The study of air-water surface tension of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was performed due to capability of the surfactant to reduce the surface tension of water. It was found that present of surfactant molecules affected the air/water surface tension as they replaced some water molecules in the surface, thus the force of attraction between surfactant and water molecules are less than those among water molecules. This results in a reduced contraction force.

Purified non-ionic surfactant was dried under vacuum at 40 °C overnight prior to the investigation. The surfactant was weighed and dissolved in distilled water at a specific concentration. Measurement of the air/ water surface tension in the presence of

methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside as surfactant was performed using De Nouy Ring method at 55 ºC. The initial surfactant solution was used to prepare a series of dilutions to obtain different concentrations. A Petri dish containing aqueous surfactant dilution was mounted on a hydraulically driven platform. The temperature of the platform was controlled at 55 °C using water circulating system. The surface tension was calculated from the maximum force on the ring (Drummond & Wells, 1998). Replications of the measurements were performed until a set of five measurements showed a standard deviation of 0.05 mN m^{-1} or less.

The ring was cleaned by flaming it prior to every measurement. The Petri dish was cleaned by soaking it in potassium dichromate solution for 5 minutes and rinsed with distilled water before every measurement. The Petri dish was checked for carry-over surfactant molecules by reading the surface tension of distilled water using the same method for surface tension measurement. The Petri dish was assumed to be free from carry-over surfactant molecules when the surface tension of the distilled water alone was found to be in the range of 70 ± 2 mN m^{-1}.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Lipase apparent activity

Lipase apparent activity was determined using *p*-nitrophenyl palmitate hydrolysis method. The multiphase reagent involves 0.2 mL of 10 mM *p*-nitrophenyl palmitate (*p*NPP) in hexane solution and 3.0 mL of 0.1 M NaOH aqueous solution. A tiny magnetic stirrer was used in the cuvette for mixing during the reaction as the reaction involved two (2) phase solution. A uniform yellow coloration in the aqueous alkaline phase was visible once *p*-nitrophenol is produced from the hydrolysis of *p*NPP. Value for *p*- nitrophenol molar extinction coefficient as calculated using equation 3.1 was 1070.43 L mol^{-1} cm^{-1}.

Hydrolysis reaction was initiated with addition of 10 mg of immobilized lipase into the *p*NPP/NaOH mixture. Four different temperatures were used (35, 43, 50 and 57 ºC) in evaluating the lipase apparent activities. The activity was calculated using Equation (3.3). The apparent activities of lipase are shown in Table 4.1:

Table 4.1: Apparent lipase activity at 35, 43, 50 and 57 °C with *p*NPP as substrate

Temperature (^{o}C)	Δ Abs $_{410nm}$ (min^{-1})	Lipase apparent activity (U/mg)
35	0.092	0.18 ± 0.04
43	0.25	0.40 ± 0.00
50	0.27	0.54 ± 0.00
57	0.30	0.59 ± 0.02

- Reaction was done in three replicates
- 1 U/mg: Hydrolysis of *p*NPP at rate of 1 μmol min^{-1} by 1 mg of lipase.

Apparent activity of immobilized lipase (CAL B) supplied from Novozyme 435 within temperature range of 35 to 57 °C was determined. It was found that the enzyme remained active within the temperature range tested. From the data (Table 4.1), it was found that the activity of lipase increased with the increased in temperature.

Activity and specificity of lipase has been known to be dependent on its environmental condition (Carrea *et al.*, 1995; Kazlauskas & Bornscheuer, 1998; Degn & Zimmermann, 2001). The increase in the hydrophobicity of the medium used for lipase catalyzed hydrolysis will assist in improving stability of the enzyme during the reaction. The hydrophobicity of solution is reflected in its log *P* value. Log *P* is a measure of the partitioning coefficient of *n*-octanol/water two phase systems. High log *P* value indicates that the solvent is highly hydrophobic. According to a few researches, the activity of lipase is correlated with the log *P* value of the medium (Laane *et al.*, 1987; Reslow *et al.*, 1987; Valivety *et al.*, 1993; Ducret *et al.*, 1998; Degn & Zimmermann, 2001). Since only one primary type of solvent was used in the apparent lipase activity measurement, therefore it can be concluded that the effect of solvent hydrophobicity towards lipase activity was consistent within all samples tested.

4.2 Quantification of methyl α-*D*-glucopyranoside as indirect assay for esterification

After the removal of the remaining glucose derivative (methyl α-*D*-glucopyranoside) from the reaction mixture, the residual methyl α-*D*- glucopyranoside was quantified using UV-Vis spectrophotometer (Jasco, Japan) through phenol sulphuric acid (PSA) catalyzed method at wavelength of 490 nm. Figure 4.1 shows the standard calibration between absorbance at 490 nm and glucose concentration, which was used as reference plot for the analysis.

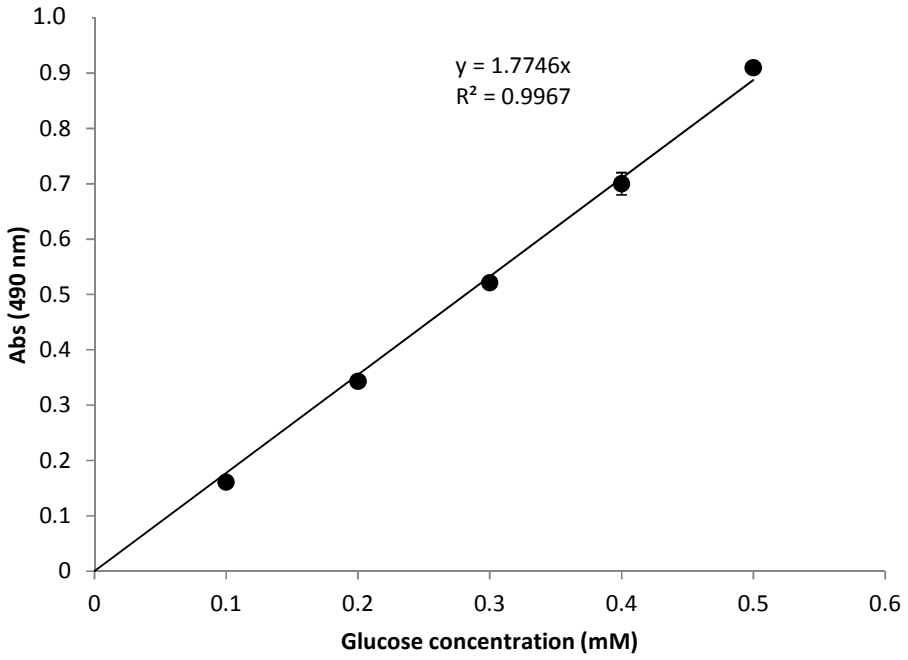


Figure 4.1: Standard calibration for glucose in 20% (w/v) salt water

From stoichiometric equation of esterification in production of carbohydrate esters, as shown previously in Chapter 2 (Figure 2.6), the formed carbohydrate ester (methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside) is a product from the reaction between 1 mol of acyl acceptor (methyl α-*D*-glucopyranoside) with 1 mol of the acyl donor. Hence, the use of methyl α-*D*-glucopyranoside residual concentration as the response parameter allows an indirect quantitative analysis of the sugar ester formed.

The phenol sulphuric acid colorimetric assay method was used for the quantification of methyl α-*D*-glucopyranoside using a UV-Vis spectrophotometer. The non-reducing end of methyl α-*D*-glucopyranoside was hydrolyzed primarily when reacted with the concentrated sulphuric acid. The reduced glucose was then oxidized and eventually reacted with phenol thus forming a yellow color. The colorimetric analysis can be measured at the same wavelength as reducing sugar (glucose). A blank of 20 % (w/v) salt water was used in all the assays as the correction as it removes the background noise from the medium used for methyl α-*D*-glucopyranoside separation. Investigations on possible interferences of hexadecanoic acid (as the co-substrate) as well as the salt water (as medium used for separating the remaining methyl α-*D*-glucopyranoside from reaction mixture) were conducted and are shown in Figure 4.2:

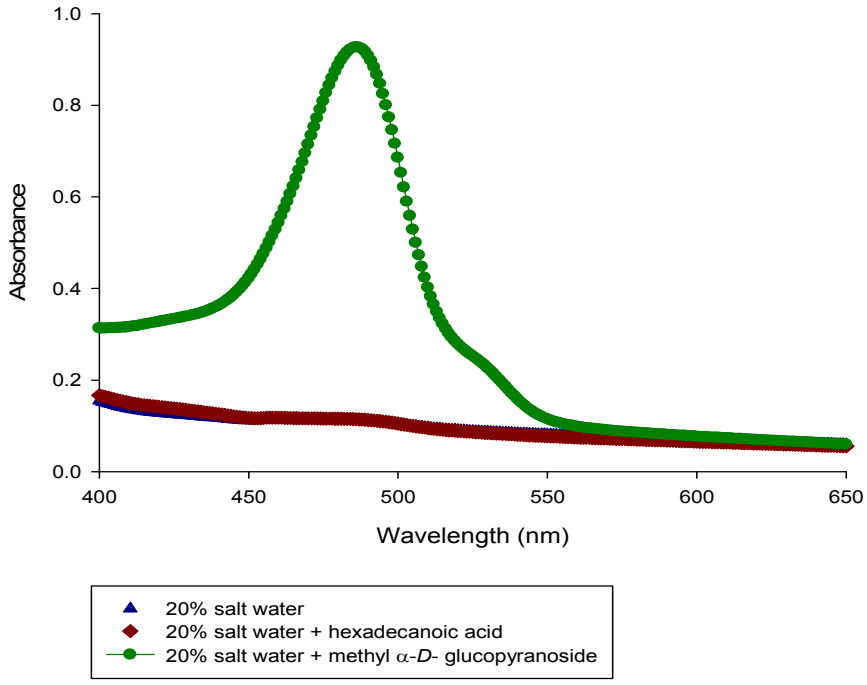


Figure 4.2: Absorption spectra of three different solutions used as mixture in methyl α-*D*-glucopyranoside quantification.

From the spectrum, it was found that both hexadecanoic acid and 20 % salt solution did not interfere with the glucose absorbance reading since the only peak found at 490 nm was for 20 % (w/v) salt water + methyl α-*D*-glucopyranoside solution. This was subsequently used for maximum absorbance wavelength in the quantification of residual methyl α-*D*-glucopyranoside.

4.3 Product purification

Products were purified using method that was assigned specifically based on its acyl donor chain length. The purification is considered successful if only one spot is visible in thin layer chromatography (TLC) (Figure 4.3). The method, however, provides only qualitative detection of the product. The product was then subjected to ^{1}H NMR for structure elucidation. Lipase has been known for its specificity towards the C-

6 moiety of glucose (Arcos *et al.*, 1998; Degn *et al.*, 1999). Based on the NMR spectrum (Figure 4.4), the enzyme lipase only catalyzed the production of monoester *viz.* reaction at the primary hydroxyl group at carbon number 6 (C-6) of the acyl acceptor (methyl α-*D*-glucopyranoside). This is depicted in the NMR spectrum (Figure 4.4) by chemical shift of C-6 for methyl α-*D*- glucopyranoside at δ 4.65 ppm.

4.4 Product (methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside ester) detection

Detection of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside in the reaction mixture and its initial identification was done by spotting the reaction mixture onto a TLC plate. Two samples were applied in the test; one spot was from the reaction mixture and the other was from a control mixture (without lipase). The spotting onto TLC plates resulted in the formation of two (2) spots with R_f of ≈ 0.66 and 0.43, one each for the reaction mixture and control (no lipase respectively). This is shown in Figure 4.3. The presence of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside ester was confirmed by NMR spectroscopy as shown in Figure 4.4.

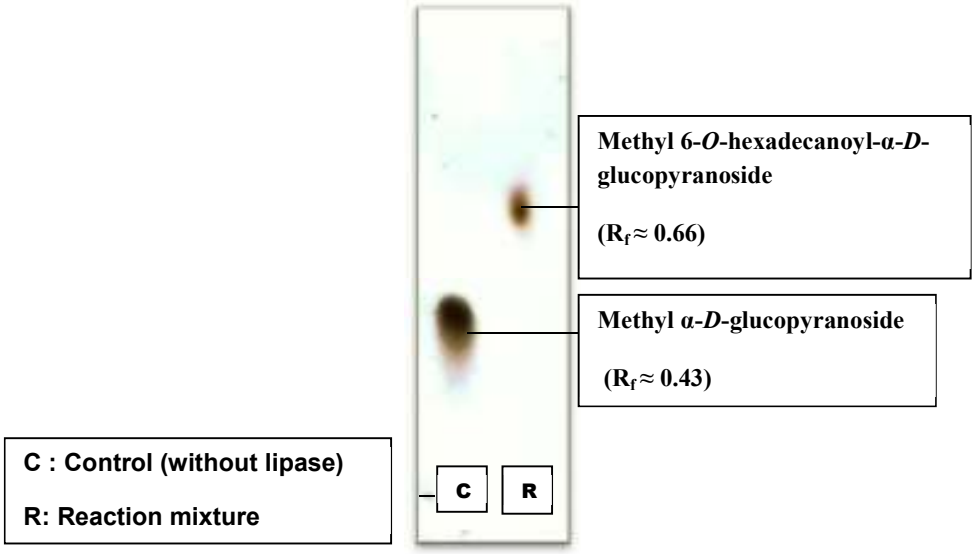


Figure 4.3: Thin layer chromatography of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside ester reaction mixture**.**

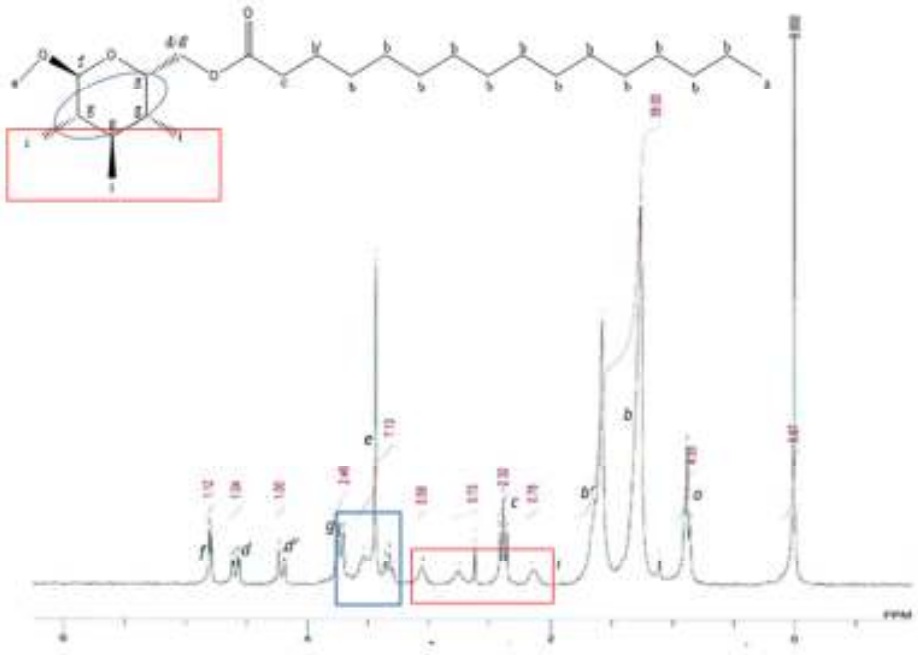


Figure 4.4: NMR spectra for sugar ester determination. Inset shows the structure of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside

Figure 4.4 showed ^{1}H NMR spectra for a partially purified reaction mixture. Spectra reading was done at 270 MHz using deuterated chloroform ($CDCl_3$) (99.8 atom.% D) as the dissolving solvent. Proton shifts were recorded in ppm relative to internal trimethylsilane (TMS) (^{1}H, δ 0.0): δ 4.80-4.78 (1H, C_1-H methyl α-*D*-glucopyranoside), 4.62-4.52 (1H, C_5-H of methyl α-*D*-glucopyranoside), 4.24-4.19 (1H, C'_6-H of methyl α-*D*-glucopyranoside), 3.76 – 3.40 (multiple H, C_2, C_3, C_4, C_5-H), 3.5 (3H, OCH), 3.0-2.20 (multiple OH, C_2, C_3, C_4-OH of methyl α-*D*-glucopyranoside), 2.40 – 2.30 (CH_2 of hexadecanoic acid functional end), 1.6 (2H, C_3 of hexadecanoic acid), 1.3 (24H of repeating unit of CH_2 hexadecanoic acid carbon atom). 0.9 (3H of hexadecanoic acid terminal end).

4.5 Full factorial experimental design

Investigation on the effects of selected process parameters towards the biosynthesis of carbohydrate ester was conducted by using full factorial experimental design (MINITAB® 15 software). A set of tri-replicates center point was also applied in order to find any curvature in the system. A total 195 number of runs (including three replicates) were generated from the design and esterification efficiency (as response parameter) was calculated using Equation 3.3 that was shown previously in chapter 3. Table 4.2 showed the total number of runs in the screening experiment and the percentage (%) of substrate conversion as the response parameter.

Due to lipase high sensitivity towards presence of water in the esterification system (initiation of hydrolysis reaction), molecular sieves 4 Å (water adsorbing component) were applied as one of the parameters used in the system. Prior to that, initial water content in the reaction system was controlled at 600 ± 20 mg/L (measured using Metrohm Karl-Fischer water titrator, Switzerland) as water content measurement

during reaction did not provide an actual water content at specific time as the molecular sieves continuously adsorb the water in the system in addition to water produced as by-product from the esterification.

Table 4.2: Full factorial screening experiment combination and percentage of acyl acceptor conversion (%)

Mole ratio (acyl donor: acyl acceptor)	**Reaction Temp (^{o}C)**	**Quantity Enzyme (g)**	**Quantity 4 Å Molecular Sieve (g)**	**Agitation Speed (rpm)**	**Incubation Time (hr)**	**Percentage Conversion (%)**
1	1	-1	1	1	-1	41
1	1	-1	-1	1	-1	38
-1	1	-1	1	1	1	46
1	1	1	-1	1	-1	18
-1	-1	1	-1	-1	-1	24
-1	-1	1	1	-1	1	12
-1	1	-1	1	-1	1	19
1	-1	-1	-1	-1	-1	16
1	-1	-1	1	-1	1	46
1	-1	1	-1	-1	-1	18
1	-1	1	1	-1	1	33
-1	-1	-1	-1	-1	1	11
1	-1	1	-1	1	-1	50
1	1	1	1	1	-1	31
-1	-1	1	1	1	-1	29
-1	-1	-1	1	-1	-1	41
1	1	-1	1	-1	-1	8
-1	1	1	-1	1	-1	48

1	-1	1	1	1	1	71
-1	1	1	1	-1	1	43
-1	-1	-1	1	1	-1	26
1	1	1	-1	-1	-1	11
1	-1	-1	-1	1	-1	29
-1	-1	1	-1	-1	1	42
-1	-1	-1	-1	1	1	37
1	1	1	-1	-1	1	78
-1	-1	-1	1	-1	1	40
-1	1	1	1	-1	-1	39
-1	-1	-1	-1	1	-1	18
1	-1	1	-1	1	1	34
1	-1	1	-1	-1	1	25
1	-1	-1	-1	1	1	23
-1	-1	1	1	-1	-1	37
1	1	-1	-1	-1	1	20
-1	-1	-1	1	1	1	24
-1	1	1	-1	-1	1	47
0	0	0	0	0	0	46
-1	1	-1	1	-1	-1	7
1	-1	1	1	1	-1	51
1	-1	1	1	-1	-1	30
1	-1	-1	1	1	1	16
1	1	-1	-1	-1	-1	12
1	1	1	-1	1	1	56
1	1	-1	1	-1	1	39
1	1	1	1	-1	-1	29
-1	1	-1	-1	-1	-1	12

-1	1	-1	-1	-1	1	32
1	-1	-1	1	-1	-1	35
1	-1	-1	1	1	-1	23
-1	1	1	-1	-1	-1	12
-1	-1	-1	-1	-1	-1	44
1	1	1	1	1	1	59
-1	1	-1	-1	1	1	19
-1	1	1	-1	1	1	30
1	1	1	1	-1	1	34
-1	-1	1	-1	1	-1	18
-1	-1	1	1	1	1	45
-1	1	-1	-1	1	-1	7
1	1	-1	-1	1	1	50
-1	1	-1	1	1	-1	12
1	1	-1	1	1	1	50
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1	-1	-1	-1	-1	1	51
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-1	-1	1	1	-1	1	17
-1	1	-1	-1	1	-1	11
1	-1	-1	-1	1	1	35
1	1	1	-1	-1	-1	8
1	-1	-1	1	1	-1	14
-1	-1	-1	1	1	-1	37

-1	-1	-1	-1	1	-1	20
1	1	1	1	1	-1	40
1	1	-1	-1	1	-1	28
1	-1	-1	-1	1	-1	26
-1	1	-1	-1	-1	-1	8
1	1	1	-1	-1	1	79
-1	1	1	-1	-1	-1	8
-1	1	1	1	-1	-1	28
1	1	1	1	1	1	70
-1	-1	1	1	-1	-1	36
1	1	-1	-1	-1	-1	18
-1	1	1	1	1	-1	57
-1	-1	-1	1	1	1	36
-1	1	-1	-1	-1	1	43
1	-1	1	1	1	1	66
-1	-1	-1	-1	-1	1	7
1	-1	1	1	1	-1	49
-1	1	-1	-1	1	1	22
1	-1	1	-1	1	1	34
-1	1	1	-1	1	1	25
-1	1	1	1	-1	1	58
1	-1	1	-1	-1	-1	20
-1	-1	1	-1	1	1	30
-1	1	1	1	1	1	48
-1	-1	-1	1	-1	1	46
-1	-1	-1	-1	1	1	27
-1	1	-1	1	1	-1	11
-1	-1	1	-1	1	-1	24

-1	1	1	-1	-1	1	43
1	1	1	1	-1	1	30
1	-1	-1	-1	-1	-1	11
-1	-1	1	1	1	-1	24
0	0	0	0	0	0	44
1	-1	1	-1	1	-1	50
1	1	-1	1	-1	1	44
1	1	1	-1	1	-1	20
1	-1	-1	1	1	1	15
1	-1	-1	-1	-1	1	33
1	1	1	-1	1	1	55
-1	-1	-1	-1	-1	-1	47
-1	1	-1	1	1	1	28
-1	1	1	-1	1	-1	38
-1	1	-1	1	-1	1	20
1	-1	1	-1	-1	1	38
1	1	1	1	-1	-1	32
1	1	-1	1	1	1	55
1	-1	1	1	-1	1	31
1	1	-1	1	-1	-1	9
1	-1	-1	1	-1	-1	32
-1	-1	-1	1	-1	-1	42
1	-1	1	1	-1	-1	37
-1	-1	1	-1	-1	-1	26
1	1	-1	-1	1	1	47
1	-1	-1	1	-1	1	37
-1	1	-1	1	-1	-1	9
1	1	-1	-1	-1	1	22

1	-1	-1	-1	1	1	30
-1	-1	1	-1	-1	-1	32
-1	1	1	1	-1	1	58
-1	-1	-1	-1	-1	1	20
-1	1	1	1	1	-1	67
-1	-1	-1	-1	1	-1	25
1	1	1	-1	1	1	71
-1	-1	1	-1	-1	1	46
-1	1	-1	-1	-1	-1	9
1	1	1	-1	1	-1	26
1	1	-1	1	-1	1	37
-1	-1	-1	1	1	1	30
1	-1	-1	1	1	1	21
1	-1	-1	-1	-1	1	39
-1	1	-1	1	1	1	36
1	-1	1	1	-1	-1	31
-1	-1	-1	-1	-1	-1	48
1	1	-1	-1	-1	1	24
-1	1	-1	-1	1	-1	10
-1	1	1	-1	-1	1	44
1	-1	-1	1	-1	1	27
1	1	-1	1	1	-1	55
1	-1	1	-1	-1	-1	15
-1	1	1	-1	1	1	25
0	0	0	0	0	0	46
1	1	-1	1	1	1	47
1	1	1	1	-1	-1	26
1	-1	1	1	1	1	50

-1	1	-1	-1	1	1	37
-1	1	1	1	1	1	45
-1	-1	1	1	1	1	53
-1	-1	1	1	-1	1	10
1	1	-1	-1	1	-1	34
-1	-1	-1	-1	1	1	17
-1	-1	-1	1	1	-1	25
1	-1	1	-1	-1	1	46
-1	-1	1	-1	1	1	26
1	-1	-1	-1	-1	-1	20
-1	-1	-1	1	-1	-1	29
-1	-1	1	1	-1	-1	31
1	-1	1	1	-1	1	47
1	-1	1	1	1	-1	50
1	-1	-1	1	1	-1	13
1	1	-1	-1	1	1	52
-1	1	1	1	-1	-1	53
-1	-1	1	-1	1	-1	21
-1	1	1	-1	1	-1	3
1	-1	1	-1	1	-1	47
1	-1	1	-1	1	1	31
1	1	1	1	1	1	73
1	1	1	1	1	-1	33
-1	1	-1	1	1	-1	17
1	1	1	-1	-1	1	78
-1	-1	1	1	1	-1	30
1	1	-1	-1	-1	-1	17
-1	1	1	-1	-1	-1	12

-1	1	-1	1	-1	1	26
-1	-1	-1	1	-1	1	37
1	-1	-1	-1	1	-1	29
-1	1	-1	1	-1	-1	11
1	1	1	-1	-1	-1	12
1	-1	-1	1	-1	-1	31
1	1	1	1	-1	1	21
1	1	-1	1	-1	-1	6
-1	1	-1	-1	-1	1	43

Due to limited solubility of methyl α-*D*-glucopyranoside in *tert.* butanol, only number of moles of acyl donor were applied as one of the parameter used in the system.

4.6 Normality test of data

Evaluation of obtained data to be applied in statistical analyses that based on normal distribution was conducted. Normality of the data was determined using Kolmogorov - Smirnov normality test. The probability plot for the response (conversion %) is shown in Figure 4.5.

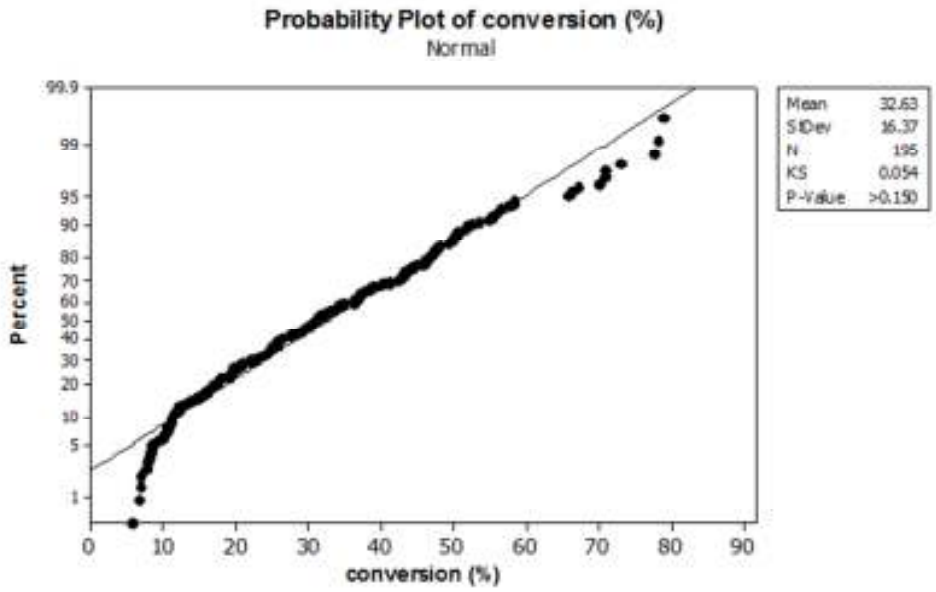


Figure 4.5: Probability plot for Kolmogorov- Smirnov normality test

From the normality analysis on the data, it was found that the *p*- value of the data was larger than 0.15. This showed that the test is not significantly different based the alpha (α) value designated for Kolmogorov – Smirnov test (α = 0.05). Therefore, the data is said to be normal and all statistical and post statistical analysis can be done according to the usual (normal) rule of tests.

4.7 Statistical analysis

Statistical analysis on the six main factors tested was performed in order to evaluate the significance of every factor tested. Analysis of Variance (ANOVA) for the above data were performed by MINITAB®15 software and the results obtained is shown in Table 4.3. The normal plot of the standardized effects at $\alpha = 0.05$ was constructed and based on the statistical analysis, only the effect of different temperatures used was found to be insignificant towards the process. The rest of the variables showed significant effects towards the reaction. This is shown in Figure 4.6.

Table 4.3: ANOVA between different parameters tested

Parameter	DF	SS	MS	*F*	*P*	Level of Significance
Mole ratio (carb: f. acid)	2	2130	1065	4.10	0.018	Yes
Reaction temp.	2	1132	566	2.14	0.121	No
Quantity enzyme	2	5663	2832	11.74	0.000	Yes
Quantity 4Å	2	2141	1070	4.12	0.018	Yes
Agitation speed	2	2432	1216	4.71	0.010	Yes
Incubation time	2	6440	3220	12.57	0.000	Yes

* DF: Degree of freedom, SS: Sum of squares, MS: Mean square, α=0.05.

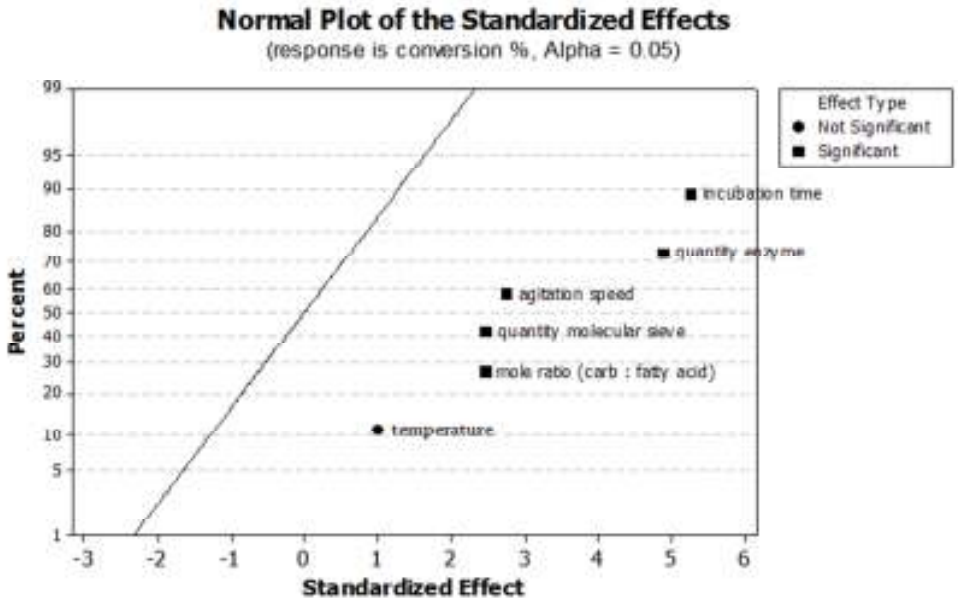


Figure 4.6: Normal plot of the standardized parameters tested at α = 0.05

4.8 Main effect analysis on the esterification reaction

The effect of individual variables tested on the bio-synthesis of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was evaluated separately using the main effect plot. All of the variables produced similar trends on their effects towards the esterification. The trend is depicted in Figure 4.7. It was found that the highest point within the range of every parameter tested resulted in the highest methyl α-*D*-glucopyranoside conversion.

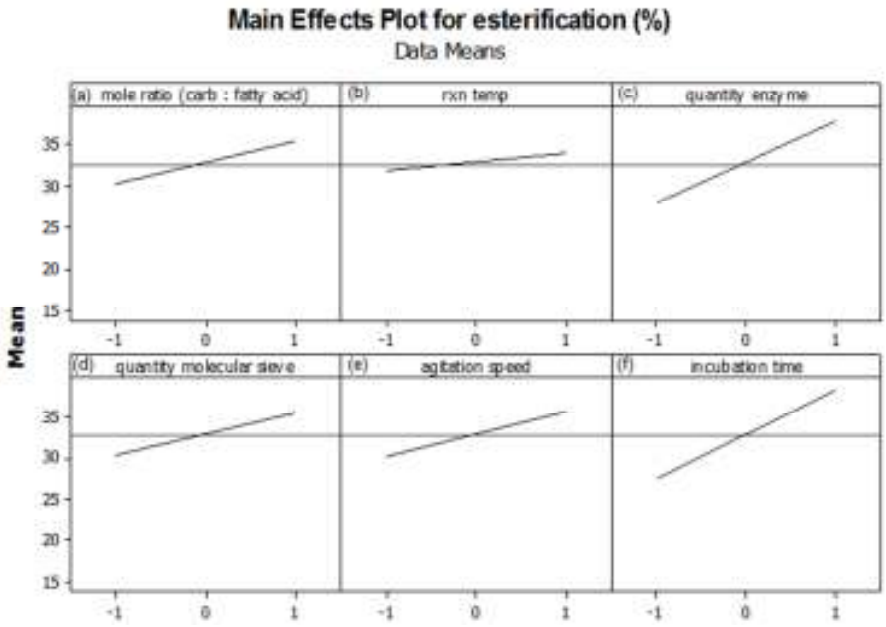


Figure 4.7: Main effect plot for synthesis of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside. (a) Effect of mole ratio (acyl donor : acyl acceptor) (mmol) (b) Effect of reaction temperature (°C), (c) Effect of enzyme loading (g), (d) Effect of 4Å molecular sieve loading (g), (e) Effect of agitation speed (rpm), (f) Effect of incubation time (hour). Representation for code variable values is shown in Table 3.1 (Chapter 3).

Figure 4.7 (a) showed the effect of two levels of mol ratio (palmitic acid: methyl α-*D*-glucopyranoside) on the mean percentage of esterification. It was observed that at mol ratio -1 (1 mol of hexadecanoic acid: 1 mol of methyl α-*D*-glucopyranoside), the esterification efficiency is significantly lower than mol ratio of 3:1. The trend generally applied for synthesis of different types of sugar esters *viz.* fructose ester (Sereti *et al.*, 1998), xylitol ester (Adnani *et al.*, 2010), sorbitol ester (Gulati *et al.*, 2003). This pattern may be due to availability of palmitic acid that became the driving substrate for the esterification as the mechanism of acyl-transfer catalyzed by lipase was shown to follow ping-pong bi-bi mechanism (Martinelle & Hult, 1995). Once the intermediate is formed, only the nucleophile substrate of carbohydrate attacks the intermediate complex thus promoting the product formation. Schematic diagram showing the esterification catalyzed by lipase is shown in Figure 4.8 (Reyes-Duarte *et al.*, 2005).

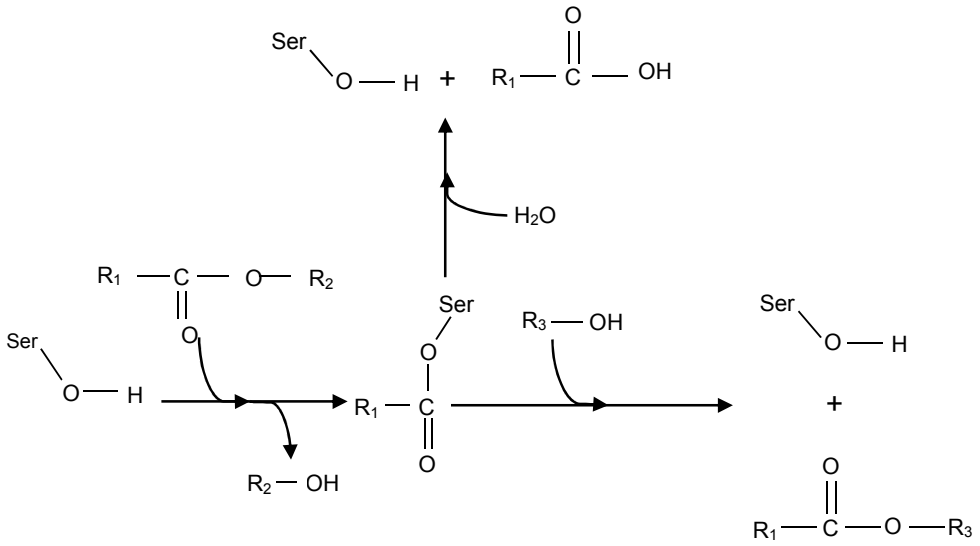


Figure 4.8: Ping pong Bi-Bi mechanism of esterification catalyzed by lipase. Ser-OH represent catalytic site serine residue (Reyes-Duarte *et al.*, 2005).

Reaction temperature however showed insignificant effect towards the methyl α-*D*-glucopyranoside conversion. Although it can be seen from Figure 4.7 (b) the mean esterification efficiency increased slightly with the increase in temperature, it is however inconclusive that this variable puts a very significant effect on the methyl α-*D*-glucopyranoside conversion within the range of temperature used in this study. This observation may be postulated from the limitation in acyl acceptor (methyl α-*D*-glucopyranoside) solubility, which is very poor. Although Figure 4.7 (b) indicates a slight increase of product formation with increasing temperature, the effect is minor. It comprises of the gradient with the statistical error clarifying the temperature as insignificant parameter.

Figure 4.7 (c) shows the effect of enzyme lipase loading (g) on the mean esterification efficiency. It can be seen that the response was higher at higher lipase loading. The trend can be explained by the increase in the availability catalytic sites (Tai & Brunner, 2009).

Excess of water in reaction medium will favor the reverse hydrolytic reaction (Yoo *et al.*, 2007; Ha *et al.*, 2010). From Figure 4.7 (d), it can be observed that the esterification efficiency increased with the increase in 4Å molecular sieve content in the medium. The presence of molecular sieves adsorbed the water molecules produced from the esterification reaction thus allowing the forward reaction to proceed longer.

Both Figure 4.7 (e) and (f) represented the influence of physical parameters *viz.* agitation speed (rpm) and incubation time (hour) respectively towards the synthesis of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside. Both of these parameters showed linear response to the esterification where at higher agitation speed as well as longer incubation time resulted in higher esterification efficiency. Higher speed resulted in better mixing between the substrate with enzyme lipase thus promoting higher probability in correct substrate orientation with the lipase active site, hence resulting in a successful catalysis. Longer incubation time may behave in similar manner where longer incubation time provided longer period for esterification process to proceed and hence, more substrate can be converted into product within the duration of time used in the study. Cauglia and Canepa (2008) showed that the solubility of the reactant *i.e.* acyl acceptor (methyl α-*D*-glucopyranoside) increased with time as more product being synthesized over time.

4.9 Hydrophilic- Lipophilic Balance (HLB) value

The optimum operating conditions for synthesizing methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was used as model condition for synthesizing carbohydrate ester with different fatty acid (acyl donor) chain length. The condition used for subsequent carbohydrate ester syntheses were acyl donor to acyl acceptor mol ratio of 3:1, 2 g immobilized CAL B per g of acyl acceptor, 1 g of new batch of 4Å molecular sieves, reaction temperature of 50 °C, 200 rpm agitation speed and 24 hours of incubation

time. HLB calculation for non –ionic surfactant is as follows as introduced by Griffin (1949) (Robb, 1996) :

$$HLB = \frac{Hydrophilic\ group\ molecular\ weight}{Total\ surfactant\ molecular\ weight} \times 20$$

(Equation 4.1)

Thus, calculation of HLB values for all carbohydrate esters synthesized in this study is shown in Table 4.4:

Table 4.4: HLB values for all carbohydrate esters used in this study

Type of carbohydrate ester	**Fatty acid carbon chain length**	**HLB Calculation**
Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside	12	$20 \times \frac{193.2}{376.52} = 10.26$
Methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside	14	$20 \times \frac{193.2}{404.57} = 9.55$
Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside	16	$20 \times \frac{193.2}{432.62} = 8.93$

The HLB value decreased with increasing chain length of the fatty acid. This reflects increase in hydrophobicity and reduced water affinity of the surfactant. As indicated in Figure 2.11 of Chapter 2 (properties of non-ionic surfactant according to their HLB value), higher HLB value of non-ionic surfactant promoted them to be more water loving compared to those with lower HLB values.

With these values determined, the properties of synthesized carbohydrate esters were further investigated to see the effects of different fatty acid chain length towards the ester properties.

4.10 Properties of synthesized carbohydrate esters

4.10.1 Thermal analysis of carbohydrate esters

4.10.1.1 Differential scanning calorimetry (DSC)

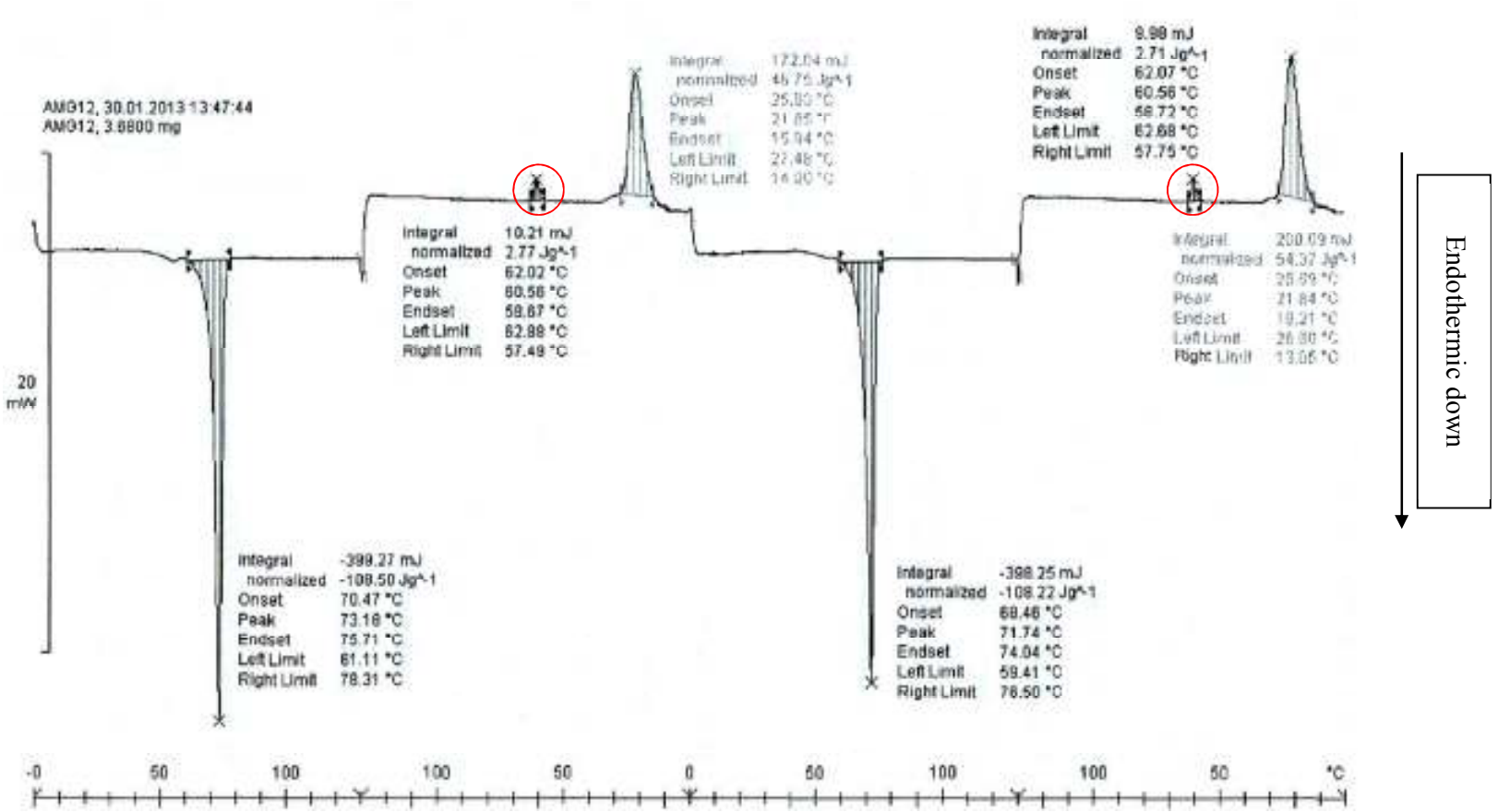
Differential scanning calorimetry (DSC) was used to monitor changes in the behaviour of synthesized carbohydrate esters carrying different fatty acid chain lengths. The carbohydrate esters that were analyzed using DSC involved methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (12-C carbohydrate ester), methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside (14-C carbohydrate ester) and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside (16-C carbohydrate ester). Upon heating the samples, the carbohydrate esters are exposed to physical stress that eventually forced the compounds to transform from solid to a liquid phase. The temperature at which the transition occurred was known as melting temperature (T_m). From the DSC curves, melting points for every carbohydrate esters can be elucidated. The endothermic melting enthalpy (ΔH_f) (J g^{-1}) provided the amount of energy required for the phase transition, e.g. melting, based on integration of peak in the curve and subsequent division with the mass of samples used for this study. Based on both T_m and ΔH_f values, the degree of increase in entropy experienced by carbohydrate esters upon phase transition (from solid to liquid) can also be quantified *via* calculating the melting entropy (ΔS_f) (J g^{-1} °C^{-1}).

This can be done using Equation (4.2) as follows:

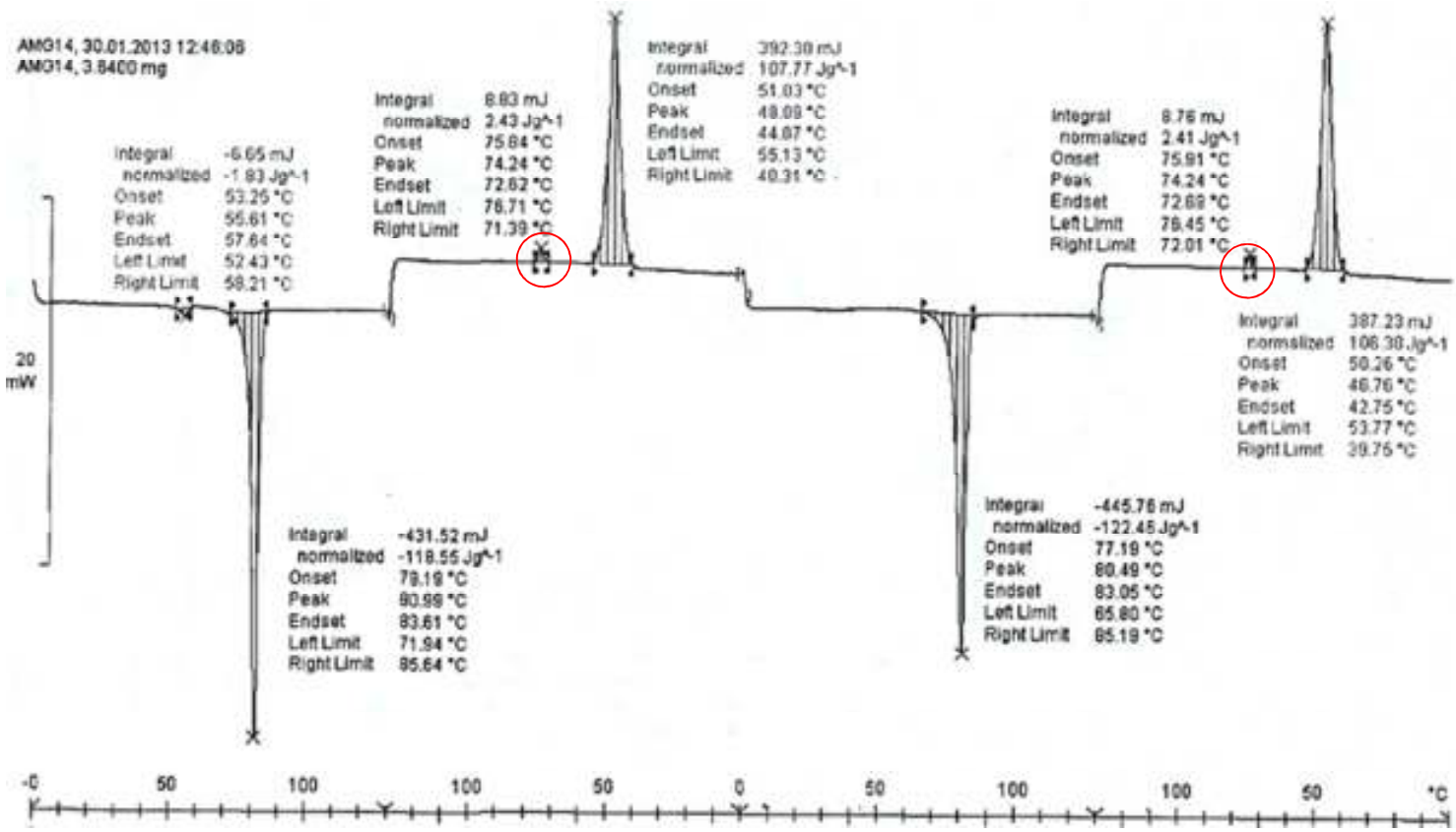
$$\Delta S_f = \frac{\Delta H_f}{T_m} \qquad \text{(Equation 4.2)}$$

DSC thermograms for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside are shown in Figure 4.9 (a), (b) and (c), respectively.

(a)


AMG12, 30.01.2013 13:47:44
AMG12, 3.6800 mg
20
mW
Integral 10.21 mJ
normalized 2.77 Jg^-1
Onset 62.02 °C
Peak 60.56 °C
Endset 58.67 °C
Left Limit 62.88 °C
Right Limit 57.49 °C
Integral 9.98 mJ
normalized 2.71 Jg^-1
Onset 62.07 °C
Peak 60.56 °C
Endset 58.72 °C
Left Limit 62.68 °C
Right Limit 57.75 °C
Integral -399.27 mJ
normalized -108.50 Jg^-1
Onset 70.47 °C
Peak 73.18 °C
Endset 75.71 °C
Left Limit 61.11 °C
Right Limit 78.31 °C
Integral -398.25 mJ
normalized -108.22 Jg^-1
Onset 68.46 °C
Peak 71.74 °C
Endset 74.04 °C
Left Limit 59.41 °C
Right Limit 76.50 °C
-0 50 100 100 50 0 50 100 100 50 °C
Endothermic down

(b)


AMG14, 30.01.2013 12:46:08
AMG14, 3.8400 mg
Integral -6.65 mJ
normalized -1.93 Jg^-1
Onset 53.25 °C
Peak 55.61 °C
Endset 57.64 °C
Left Limit 52.43 °C
Right Limit 58.21 °C
Integral 6.83 mJ
normalized 2.43 Jg^-1
Onset 75.84 °C
Peak 74.24 °C
Endset 72.62 °C
Left Limit 78.71 °C
Right Limit 71.39 °C
Integral 392.39 mJ
normalized 107.77 Jg^-1
Onset 51.03 °C
Peak 48.09 °C
Endset 44.87 °C
Left Limit 55.13 °C
Right Limit 40.31 °C
Integral 6.76 mJ
normalized 2.41 Jg^-1
Onset 75.91 °C
Peak 74.24 °C
Endset 72.69 °C
Left Limit 78.45 °C
Right Limit 72.01 °C
Integral 387.23 mJ
normalized 106.30 Jg^-1
Onset 50.26 °C
Peak 46.76 °C
Endset 42.75 °C
Left Limit 53.77 °C
Right Limit 39.75 °C
Integral -431.52 mJ
normalized -118.55 Jg^-1
Onset 79.19 °C
Peak 80.99 °C
Endset 83.61 °C
Left Limit 71.94 °C
Right Limit 85.64 °C
Integral -445.76 mJ
normalized -122.45 Jg^-1
Onset 77.19 °C
Peak 80.49 °C
Endset 83.05 °C
Left Limit 65.80 °C
Right Limit 85.19 °C
20 mW
-0
50
100
100
50
0
50
100
100
50
°C
Endothermic down

(c)

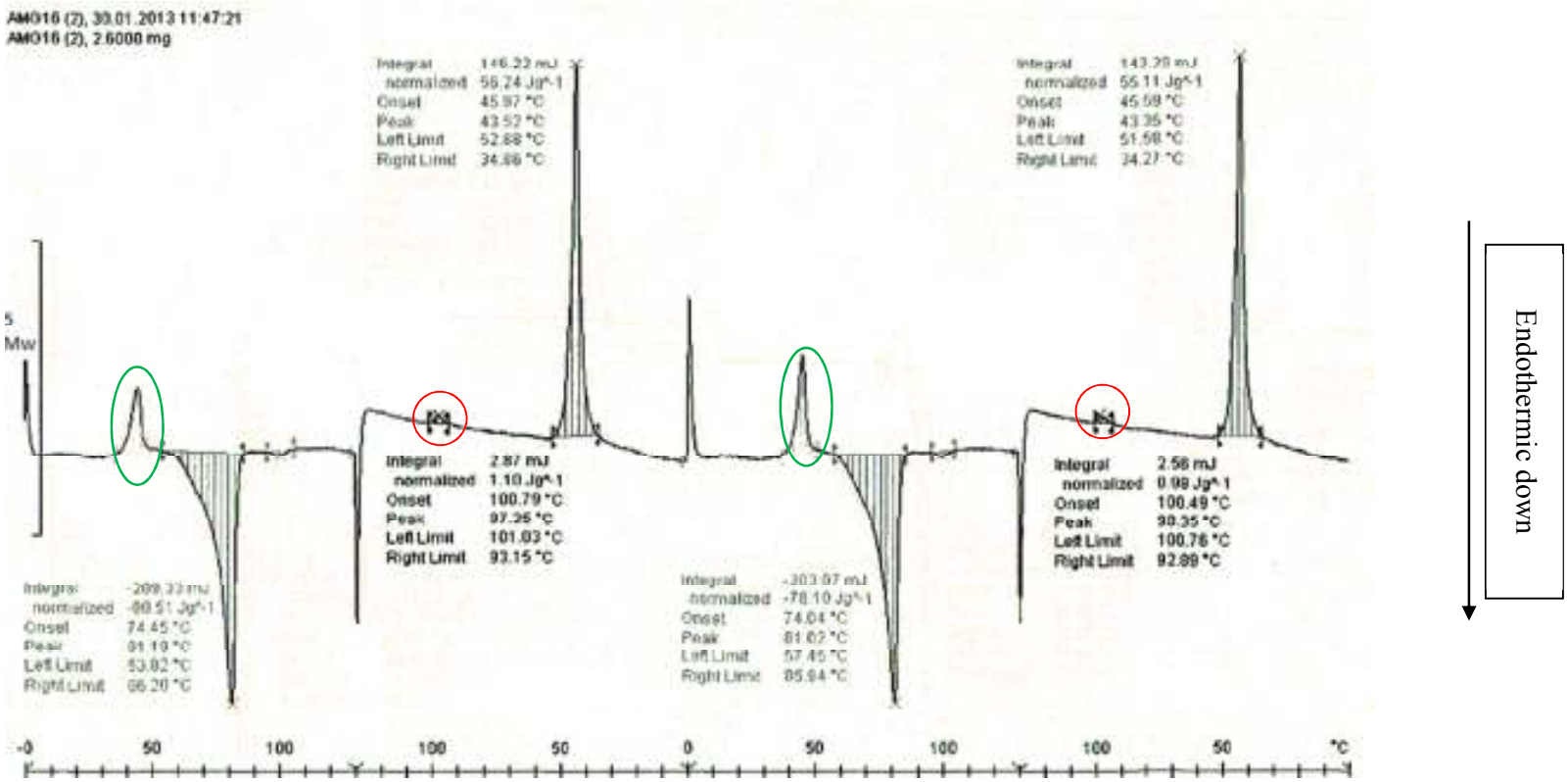


Figure 4.9: DSC thermograms for (a) methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, (b) methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and (c) methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside.○ represents crystallization peak and ○represents the mesophase transition peak.

Values for melting temperature (T_m), endothermic melting enthalpy (ΔH_f) and entropy of melting (ΔS_f) for all of the carbohydrate esters tested are shown in Table 4.5.

Table 4.5 : Melting temperature (T_m), Crystallization temperature (T_c), endothermic melting enthalpy (ΔH_f) and entropy of fusion (ΔS_f) for all of carbohydrate esters tested.

Carbohydrate ester	**Melting temperature (T_m) (°C)**	**Crystallization temperature (T_c) (°C)**	**Endothermic melting enthalpy (ΔH_f) (kJ mol^{-1})**	**Entropy of melting (ΔS_f) (kJ mol^{-1} °C^{-1})**
Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside	73.2	21.7	40.9	0.55
Methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside	80.1	48.1	48.0	0.60
Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside	81.2	43.5	34.8	0.43

Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside has the lowest melting temperature with 73 °C followed by methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside (80 °C) while the longest chained surfactant methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside exhibited the highest melting point at 81 °C. The trend is consistent with increasing melting points for increasing chains of saturated fatty acids. While the melting temperature increased significantly from 12-C to 14-C it appears to reach a plateau at higher chain length, because the 16-C differs only slightly from the 14-C carbohydrate ester. This behavior is in line with reported observations that indicated

constant melting temperatures occured for monosaccaharides with alkyl chain of C_{14} – C_{16} (Singh & Jayaraman, 2009).

Melting enthalpy was found to increase with increase in the fatty acid alkyl chain length from 12 carbons to 14 carbons. Low HLB value of synthesized carbohydrate ester (due to elongation of alkyl chain length) resulted in higher melting temperature of the ester (Szűts *et al.*, 2007). The trend however showed otherwise when hexadecanoic acid (16-C) fatty acid was used as the acyl donor. Presence of exothermic peak preceding the endothermic peak (melting) (O) indicates there was an occurrence of partial crystallization process. Thus, it is postulated that melting of the methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside (indicated by endothermic peak right after the partial crystallization process) is incomplete thus resulting in lower melting enthalpy of the ester. The melting entropy (ΔS_f) for all carbohydrate esters synthesized showed positive values thus indicating that during melting, the particles experienced a higher degree of system randomness.

All synthesized carbohydrate esters are thermally stable upon melting, as the melting temperatures recorded for the first and second heating cycle do not differ significantly. This is shown in Table 4.6.

Table 4.6: Comparison on melting temperature from 1st and 2nd heating cycles

Carbohydrate ester	**Melting temperature (T_m) (°C) (1st cycle)**	**Melting temperature (T_m) (°C) (2nd cycle)**
Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside	73	72
Methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside	80	81
Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside	81	81

Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside recorded the lowest crystallization temperature at 22 °C, while methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside crystallized at highest temperature of 48 °C. The longest chained surfactant methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside recorded a slightly lower crystallization temperature which was at 44 °C. In general it is postulated that the temperature for crystallization follows the trend of the melting temperature. Deviations may reflect kinetic obstacles, which increase with the chain length.

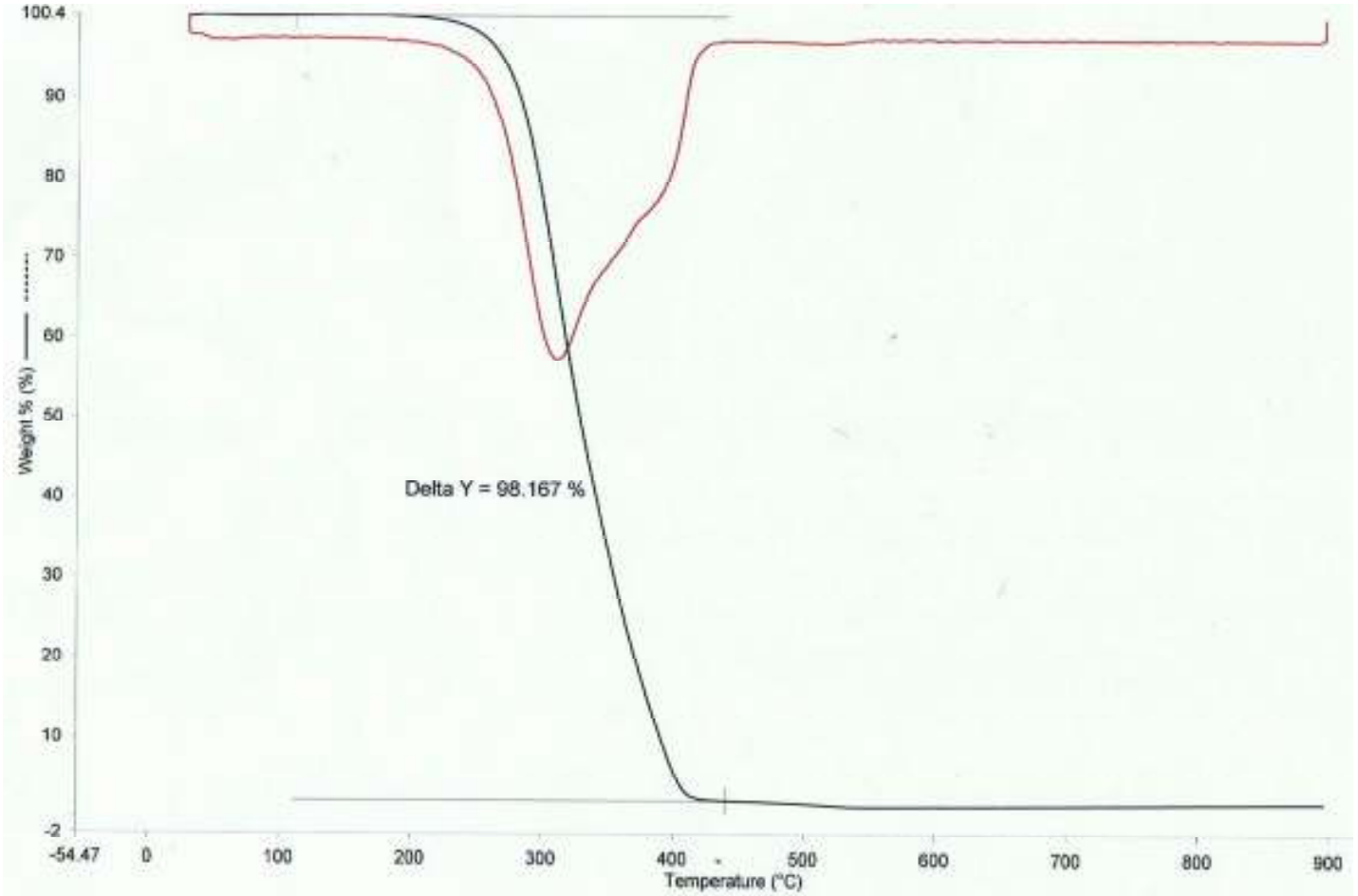
The DCS thermograms of all three carbohydrate esters indicated the presences of a metastable liquid crystal phase. Related phase transitions are shown in the red circles (○) in Figure 4.9. Transitions were only observed upon cooling, because the melting temperature of the crystal is higher than the liquid crystal clearing temperature. The liquid crystalline phase appears as an exothermic peak above the crystallization due to a kinetic hindrance of the latter as a metastable phase, which transforms over time into the more stable crystal. The liquid crystalline state is a distinct state of matter that can be observed in between the crystalline solid phase and the isotropic liquid phase (Demus *et al.*, 1998). The state, which is also called mesophase, is induced by temperature and,

hence, termed as a thermotrophic phase (Baron, 2001). Due to the metastable character of the liquid crystalline phase for the investigated carbohydrate esters, it could not be studied by optical polarized microscopy (OPM, to be discussed in sub-section 4.10.2). Presences of liquid crystal phase in all synthesized carbohydrate esters indicate that the products may find industrial applications for optoelectronics or exhibit interesting mechanical properties (Goodby *et al.*, 2007).

4.10.1.2 Thermogravimetric analysis (TGA)

All synthesized carbohydrate esters were investigated by TGA in order to study the thermal stability of the esters. This way the decomposition temperatures as well as the decomposing patterns for carbohydrate esters were determined. The thermal behaviour of synthesized carbohydrate esters was examined using a Perkin-Elmer TGA 4000 instrument from 30 °C to 900 °C at heating rate of 10 °C min^{-1}. Figure 4.10 (a), (b) and (c) show the thermograms for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside, respectively. The data were recorded as thermograms of weight (%) versus temperature (°C). The degree of mass loss is represented by delta Y.

(a)


100.4
90
80
70
60
50
40
30
20
10
-2
Weight % (%)
Delta Y = 98.167 %
-54.47
0
100
200
300
400
500
600
700
800
900
Temperature (°C)

(b)


100.3
90
80
70
60
50
40
30
20
10
-2
Weight % (%)
Delta Y = 44.766 %
Delta Y = 52.783 %
Delta Y = 1.647 %
-49.57
0
100
200
300
400
500
600
700
800
900
Temperature (°C)

(c)

Figure 4.10: TGA thermograms for (a) methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, (b) methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and (c) methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside.

Figure 4.10 (a) shows the TGA-curve for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside. The surfactant is thermally stable up to 200 °C, as no significant mass loss is recorded below this temperature.

The degradation pattern of the carbohydrate esters changes with increasing fatty acid chain length from 12 carbons to 14 carbons. This is shown in Figure 4.10 (b). Instead of only one decomposing peak (Figure 4.10 (a)), two peaks at different temperature were observed, (see Figure 4.10) (b). 45% of mass loss was observed during the first decomposition temperature (200 °C to 350 °C) while another 53% of mass loss was recorded as temperature increased from 350 °C to 450 °C. The profile got sharper when methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was applied in the analysis (Figure 4.10 (c)). 49 % of mass loss was recorded between 170 °C and 340 °C, while additional 45% of mass loss when temperature was observed at elevated temperature, between 340 °C and 440 °C.

The lower decomposition (between 170 to 350 °C) region indicates the decomposition of the hydrophilic head of the carbohydrate ester. Decomposition of methyl α-*D*-glucopyranoside was initiated by cleavage between the methoxy and glycosyl group leading to the release of methanol. The glucosyl group will then condense with other sugar molecules followed by fragmentation of the glycan into various compounds of lower molecular mass (McGinnis & Parikh, 1973). The clear decomposition region at higher temperature (340 to 450 °C) recorded for carbohydrate esters with longer fatty acid chain length (14-C and 16-C) were due to the decomposition of the released carboxylic acids. Further increase in temperature showed gradual weight loss until the weight reached a constant weight, which represented the carbonization process (Tosh, 2011).

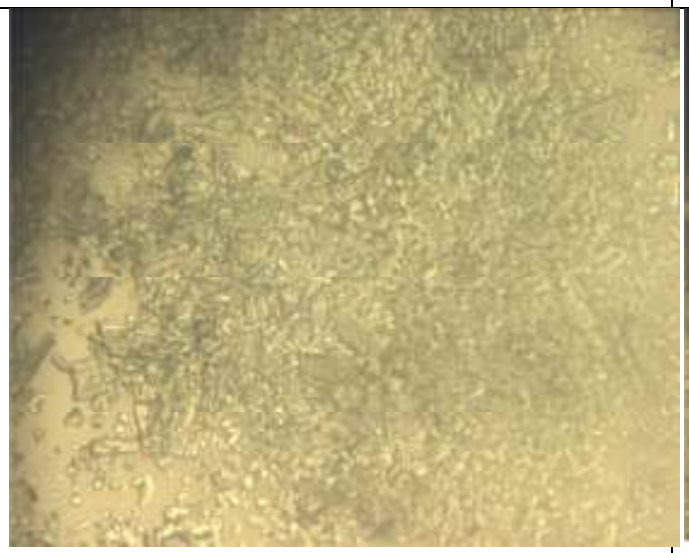
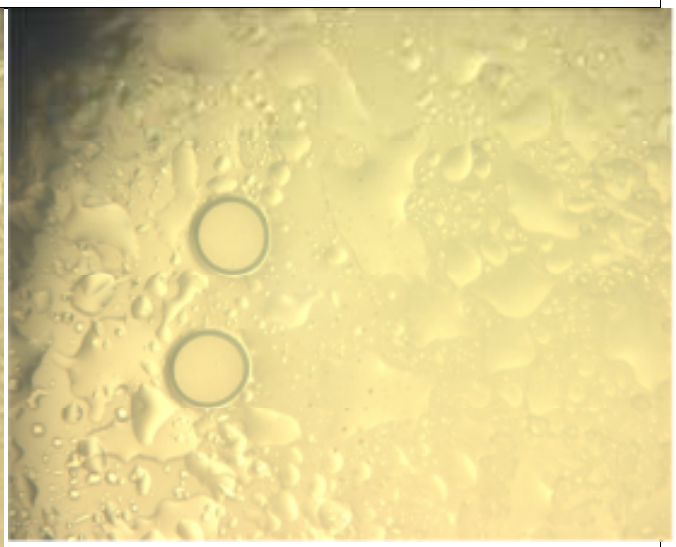
4.10.2 Optical polarized light microscopy

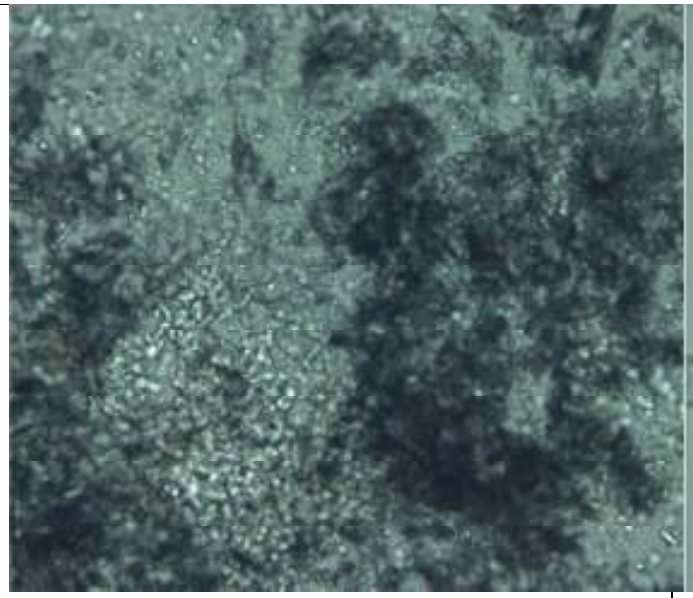
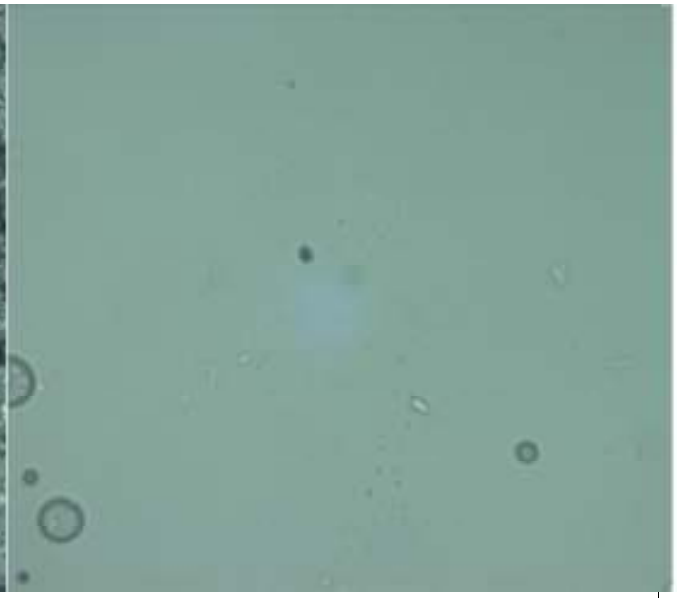
Optical polarized light microscopy (OPM) was used to record images of textures for pure carbohydrate esters upon changing temperature. This investigation enabled the estimation of melting points. The data was subsequently compared with those obtained from DSC. Apart from that, the presence and type of liquid crystals of carbohydrate esters can be validated from the OPM analyses, if the liquid crystalline phase is sufficiently stable to provide good textures.

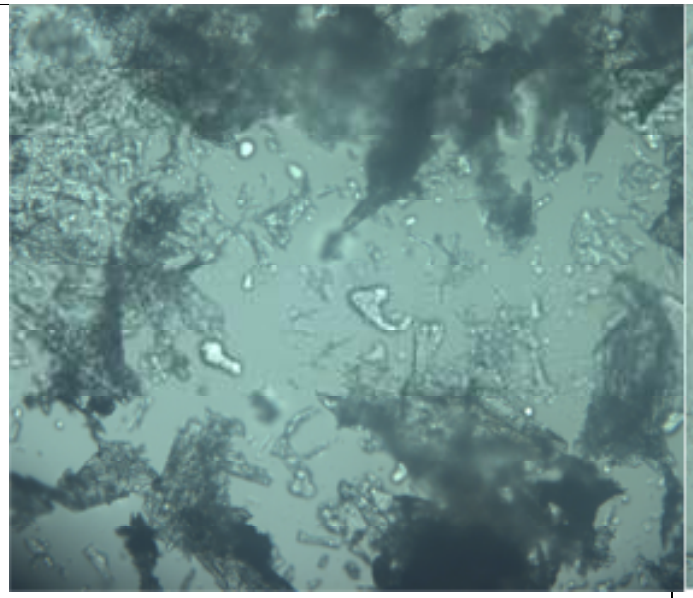
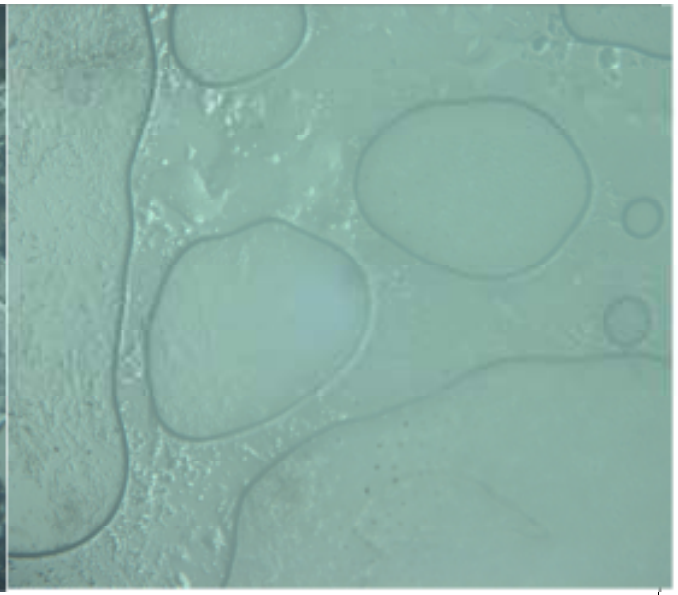
4.10.2.1 Comparison of carbohydrate esters melting temperature

The transition from the solid to the liquid phase with increase in temperature can be seen using OPM. Upon heating the carbohydrate ester, hydrocarbons first started to melt thus losing their three dimensional structure. This can be observed as the melting point. Upon reaching the clearing point, the hard carbohydrate polar head started to melt to form the isotropic liquid at a higher temperature (Abeyrathne *et al.*, 2012). Textures of pure carbohydrate esters at various temperatures are shown in Table 4.7.

Table 4.7: Solid and liquid phases for all carbohydrate esters tested

Type of Carbohydrate Ester	**Solid**	**Liquid**
Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside	 Temperature : 70 °C	 Temperature : 74 °C

Methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside		
	Temperature : 80 °C	Temperature : 82 °C

Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside		
	Temperature : 80 °C	Temperature : 86 °C

Comparison between qualitative measurement for melting temperature for all carbohydrate esters and quantitative measurement for melting points obtained from DSC is shown in Table 4.8.

Table 4.8: Comparison in melting temperatures of all carbohydrate esters between DSC and OPM

Carbohydrate ester	OPM (°C)	DSC (°C)
Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside	74	73
Methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside	80	80
Methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside	84	81

From Table 4.8, it can be seen that that the melting temperatures obtained from DSC and OPM for all carbohydrate esters studied are in good agreement with each other.

Presence of liquid crystal properties in the synthesized carbohydrate ester enhances the versatility in the applications of the product. Such functionality enhances the optoelectronic and mechanical properties of the synthesized carbohydrate esters (H. L. Razafindralambo *et al.*, 2012). Thermotrophic liquid crystal phases of mono-substituted carbohydrate ester synthesized were investigated using thermo-microscopy under crossed polarizers. The visible texture observed resulted from the defects in the carbohydrate ester's orientation. Carbohydrate monoesters exhibits only one liquid crystal phase. Due to separation of hydrophobic and hydrophilic molecular parts, a multilayer is formed. (Hashim *et al.*, 2006; Molinier *et al.*, 2006; Singh & Jayaraman, 2009; Abeyrathne *et al.*, 2012; H. L. Razafindralambo *et al.*, 2012). The texture of carbohydrate monoester (*i.e*. methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside) is shown in Figure 4.11. No liquid crystal was observed in other carbohydrate monoesters

synthesized due to higher thermostability of crystalline phase which exceeds the stability of the liquid crystal phase.

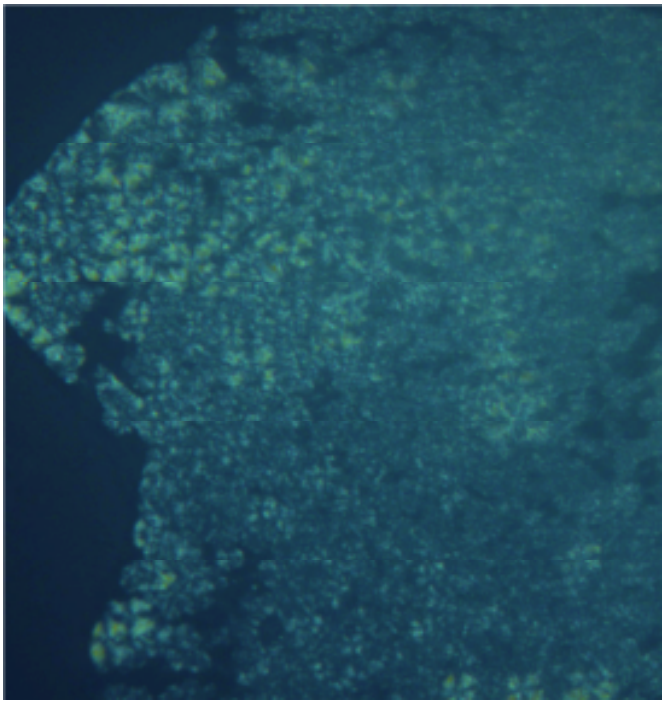


Figure 4.11: Liquid crystal texture for methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside observed at 20 X magnification under optical polarized light microscope (OPM).

4.10.3 Pseudoternary phase diagram development

Development of pseudoternary phase diagram was done using water as response parameter. A mixture of *n*- hexadecane and *n* – butanol was held constant at 1:1 weight ratio. Weight of carbohydrate ester of different fatty acids chain length was varied from 1: 10 (carbohydrate ester: oil mixture) to 10: 0 (carbohydrate ester: oil mixture). The study was performed in a thermostated waterbath of 60 °C. Water was titrated at 5 µl into the reaction mixture at every interval and was vigorously vortexed and let stand to observe the solubility before addition of the next batch of water at the same volume. It was observed that precipitation occurred when reaction mixture was left at room temperature. Reactions therefore were conducted at elevated temperature.

4.10.3.1 Pseudoternary phase diagram development for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside

Weight ratio for every component of reaction mixture is shown in Table 4.9. The data from weight fractions of oil mixture, water and methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (%) were used for pseudoternary phase diagram development in order to delineate the cutting point between a monophasic and biphasic region within the reaction mixture. The diagram is shown in Figure 4.14.

Table 4.9: Weight (g) of different components in reaction mixture for pseudoternary phase diagram development

Ratio (carbohydrate ester:oil mixture)	Carbohydrate ester (C-12) (g)	Oil mixture (*n*-hexadecane: *n*-butanol 1:1) (g)	Water (g)	Oil Weight fraction (%)	Carbohydrate ester weight fraction (%)	Water weight fraction (%)	Total weight (g)
0 : 10	0.000	0.040	0.005	90	0	10	0.045
1 : 9	0.004	0.040	0.015	68	7	26	0.059
2 : 8	0.010	0.040	0.010	67	17	17	0.060
3 : 7	0.018	0.040	0.030	45	21	34	0.088
4 : 6	0.013	0.020	0.030	32	21	48	0.063
5 : 5	0.024	0.020	0.015	34	41	25	0.059
6 : 4	0.030	0.020	0.025	27	40	33	0.075
7 : 3	0.048	0.020	0.020	23	55	23	0.088
8 : 2	0.084	0.020	0.010	18	74	9	0.114
9 : 1	0.180	0.020	0.005	10	88	2	0.205
10 : 0	0.002	0.000	0.000	0	100	0	0.002

It was observed that the solution became semi- solid (gel) at 60 °C (Figure 4.12) at weight ratio of carbohydrate ester to oil mixture (9:1). A comparison between gelated and liquid form of reaction mixtures is shown in Figure 4.13.

Figure 4.12: Formation of gel (high viscosity) of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside at weight ratio of 9:1 to the oil mixture**.**

Figure 4.13: The difference between lower ratio of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (a) to the oil mixture, and (b) to the gelated reaction mixture.

It was observed that the reaction mixture of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside: oil mixture (*n* – hexadecane: *n* – butanol) remained in liquid form when the ratio of mixture used was from 0:10 to 8:2 (w/w). The solution, however, turned into semi-solid (gelated) when the first 5 mg of water was added into reaction mixture with a ratio of 9: 1 (methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside: oil mixture). Gelation was also observed when methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was used alone in the reaction without the addition of water (at 60 °C) (ratio of carbohydrate ester to oil mixture at 10:0). The solution forms a gel at high concentration due to the formation of a self-assembled structures of surfactant (*i.e*: carbohydrate ester) or known as micelle (Szűts *et al.*, 2010). Micellization of the ester results in changing of their viscoelastic properties thus making the reaction mixture became more viscous. As elevated temperature of 60 °C was used in the phase diagram development, the high temperature favoured the dissociated hydrogen bonds between carbohydrate ester and water which would then resulted in the self-assembly of the surfactant (carbohydrate ester) amongst themselves (Berjano *et al.*, 1993).

From Figure 4.14, the highest percentage of dissolved water (52 %) was achieved at 13% methyl α-*D*-glucopyranoside and 35 % of oil mixture (*n*- butanol: *n*-hexadecane). Ratio of surfactant (methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside) to the oil mixture at the highest amount of water dissolved in the solution was approximately 2:3 (w/w), respectively. The curve line in the pseudoternary phase diagram indicates the separating barrier between monophasic (1-phase) region and biphasic region.

The ability of water to dissolve in reaction mixture drops drastically as ratio of surfactant to oil mixture increased beyond 7: 3 respectively. This is due to the tendency of surfactant to form micelle as it surpasses its critical micellar concentration thus forming micelle which can be seen physically from the gelation of the surfactant (Figure 4.14).

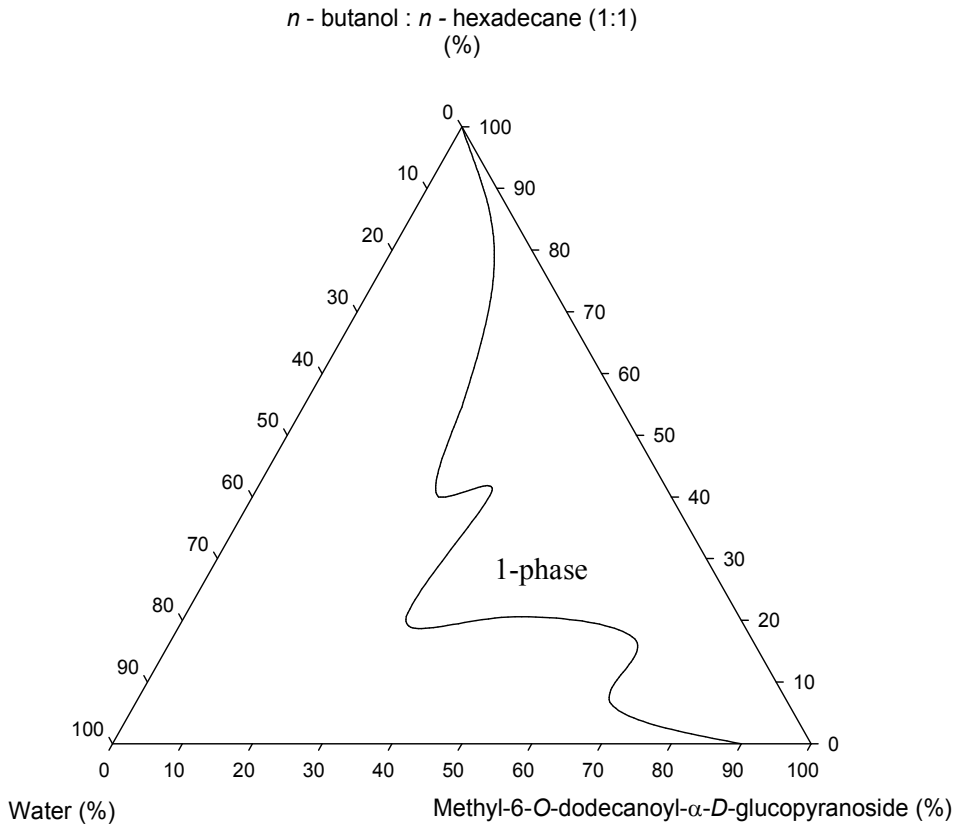


Figure 4.14: Pseudoternary phase diagram using *n*–hexadecane as model oil, *n*- butanol as co-solvent, methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside as carbohydrate ester and water as response parameter. 1-phase indicates the monophasic region of the solution**.**

4.10.3.2 Pseudoternary phase diagram development for methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside

Percentage of dissolved water within oil and surfactant mixture solution is shown in Table 4.9. It was found that oil - carbohydrate ester mixture gets gelated even at lower carbohydrate ester/ oil mixture weight ratio compared to when methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (C-12) was used as the carbohydrate ester. The solution gets gelated at 8: 2 (carbohydrate ester: oil mixture) weight ratio after first drop of water was added into reaction mixture whilst the observed ratio was recorded at 9:1 (carbohydrate ester: oil mixture) weight ratio for C-12 carbohydrate ester.

Longer carbon backbone of fatty acid chain (acyl group donor) that made up the carbohydrate ester (C-14) resulted in the increase in its surface activity (activity in lowering the surface tension of the two different solutions that are in contact with each other). The surface activity depends on the balance between the hydrophilic and hydrophobic properties of the surfactant. The relationship between hydrocarbon chain length (hydrophobic region) and surface activity is expressed by Traube's rule. The rule which was first proposed by Traube (1891) indicates that for every extra $-CH_2$ group in a compound, one requires 3 times less of the compound to produce the same lowering surface tension. As only weight of carbohydrate ester was used as manipulated parameter without taking into account the difference in molecular weight for every different carbohydrate ester, thus the number of moles in every carbohydrate ester was also different. Hence, CMC value of carbohydrate ester with longer repetition of methylene groups was predicted to be lower than shorter carbohydrate ester. This resulted in micellization of methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside (C-14 ester) at lower carbohydrate ester: oil mixture weight ratio as compared to methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (C-12 ester).

Table 4.10: The breakdown of reaction mixture (g) for pseudoternary phase diagram development

Ratio (carbohydrate ester:oil mixture)	**Oil mixture (*n*-hexadecane: *n*-butanol 1:1) (g)**	**Carbohydrate ester (c-14) (g)**	**Water (g)**	**Oil Weight fraction (%)**	**Carbohydrate ester weight fraction (%)**	**Water weight fraction (%)**	**Total weight (g)**
0 : 10	0.040	0.000	0.005	90	0	10	0.045
1 : 9	0.040	0.004	0.015	68	7	25	0.059
2 : 8	0.040	0.010	0.010	67	17	17	0.060
3 : 7	0.040	0.018	0.030	45	21	34	0.088
4 : 6	0.040	0.008	0.030	35	14	52	0.058
5 : 5	0.040	0.024	0.015	34	41	25	0.059
6 : 4	0.040	0.030	0.025	27	40	33	0.075
7 : 3	0.040	0.048	0.020	23	55	23	0.088
8 : 2	0.040	0.168	0.005	19	79	2	0.213

From the diagram (Figure 4.15), the highest percentage of dissolved water (35%) was achieved at 16% methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and 49 % of oil mixture (*n*-butanol: *n*-hexadecane). Ratio of used surfactant (methyl 6-*O*-tetradecanoyl- α-*D*-glucopyranoside) to the oil mixture at the highest amount of water dissolved in the solution was found at approximately 1: 3 (w/w).

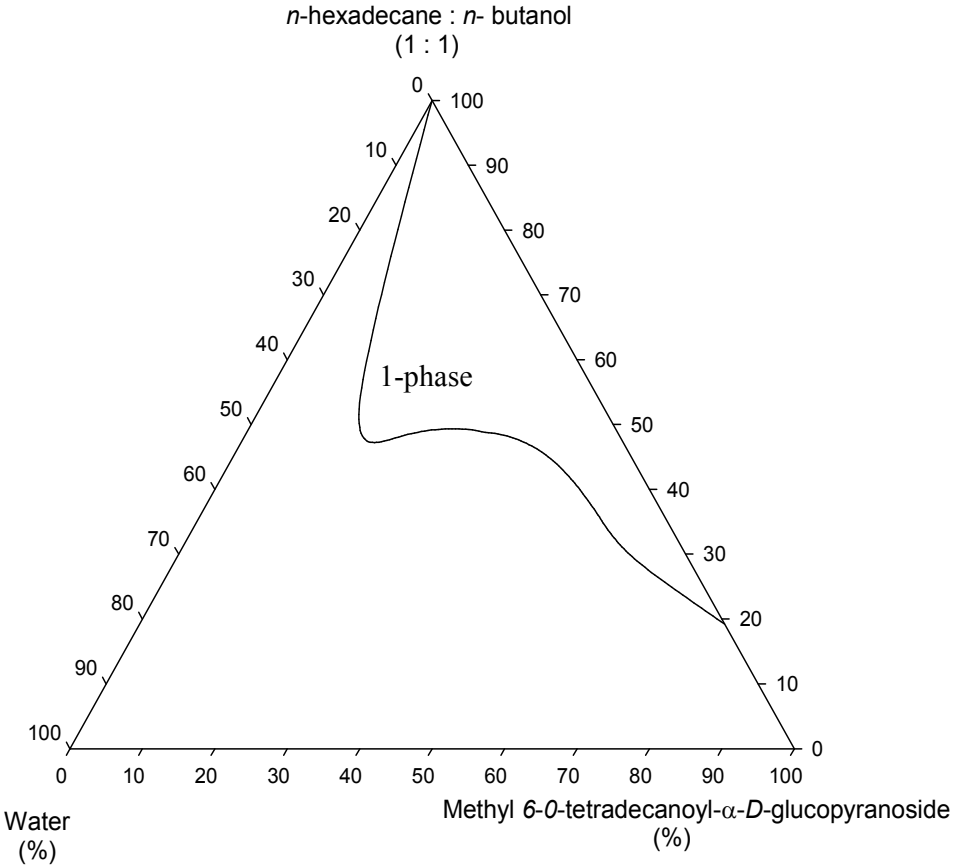


Figure 4.15: Pseudoternary phase diagram using *n* – hexadecane as model oil, *n*- butanol as cosolvent, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside as carbohydrate ester and water as response parameter. 1-phase indicates the monophasic region of the solution**.**

4.10.3.3 Pseudoternary phase diagram development for methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside (C-16)

Surfactant- oil mixture solution turned turbid instantly when the first drop of water was added at the ratio of surfactant to oil mixture 1: 9 (w/w). Long chain of hydrophobic region within the methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside backbone (C-16) decreased the polarity of the carbohydrate ester thus discontinuing the one phase region (surfactant/oil mixture/water) at lower water volume. This is also in good agreement with the HLB value of methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside where it has the lowest HLB value among all three carbohydrate esters tested (Table 4.5).

Due to the discontinuity of one phase region at the first point in the addition of water, the methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside is seen to be inapplicable as surfactant in oil/water emulsion system. Appearance of the solution when the first 0.005 mL distilled water was added is shown in Figure 4.16.

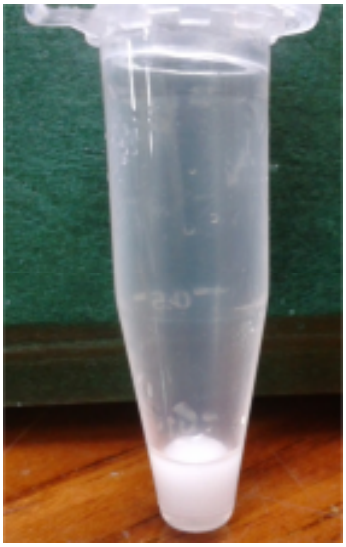


Figure 4.16: Turbidity of surfactant/ oil mixture/ water solution after addition of 5 µL of distilled water**.**

4.10.4 Air/ water surface tension study ($\gamma_{a/w}$)

This study was not performed with methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside due to solubility limitation thus resulting in inadequate data for finding an accurate CAC point. This is in agreement with Traube's rule where elongation of the chain by two -CH_2 groups resulted in the decrease of CAC by a factor of 10 (Lange & Jeschke, 1987). Absence of co-solvent (*n*–butanol used in phase diagram development) in this study further reduced the solubility of carbohydrate esters with longer fatty acid chains. It was found that only methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (C-12 fatty acid chain) dissolved sufficiently in water (55 °C) to determine the critical aggregation concentration.

Hence, the study on the air/water surface tension was performed only on methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside at elevated temperature of 55 °C. From the study, the critical aggregation concentration of the surfactants (CAC), the surface tension of surfactants at the CAC (γ_{cac}) and minimum surface tension (γ_{min}) were determined directly from the surface tension plot. Knowledge of these values allow further investigation on the surfactant air/water surface tension by applying them into formulas that calculate of the surface excess area occupied per molecule of surfactant and the Gibbs free energy of micellization (ΔG_{mic}) in kJ mol^{-1}.

The CAC value of the surfactant was identified as the surfactant concentration (C) when the gradient ($d\gamma/d \log C$) abruptly changed from maximum value to a smaller value (Clint, 1975). Experimental values for the surface tension reduction with the change of different surfactant concentrations are shown in Table 4.11. The plot of water surface tension against the surfactant concentration is shown in Figure 4.17.

Table 4.11: Air/water surface tension in the presence of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside.

	Concentration (mol L^{-1})	Log (Concentration)	Surface tension (mN m-1)	Concentration (mol L^{-1})
Surfactant (methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside)	2.14×10^{-3}	-2.67	26	2.14×10^{-3}
	1.43×10^{-3}	-2.85	26	1.43×10^{-3}
	1.34×10^{-3}	-2.87	26	1.34×10^{-3}
	6.69×10^{-4}	-3.17	26	6.69×10^{-4}
	2.68×10^{-4}	-3.57	35	2.68×10^{-4}
	1.34×10^{-4}	-3.87	43	1.34×10^{-4}
	5.36×10^{-5}	-4.27	49	5.36×10^{-5}
	2.68×10^{-5}	-4.57	58	2.68×10^{-5}
	1.34×10^{-5}	-4.87	63	1.34×10^{-5}
	5.36×10^{-6}	-5.27	65	5.36×10^{-6}
	2.68×10^{-7}	-5.57	66	2.68×10^{-7}
	0.27×10^{-7}	-6.57	66	0.27×10^{-7}

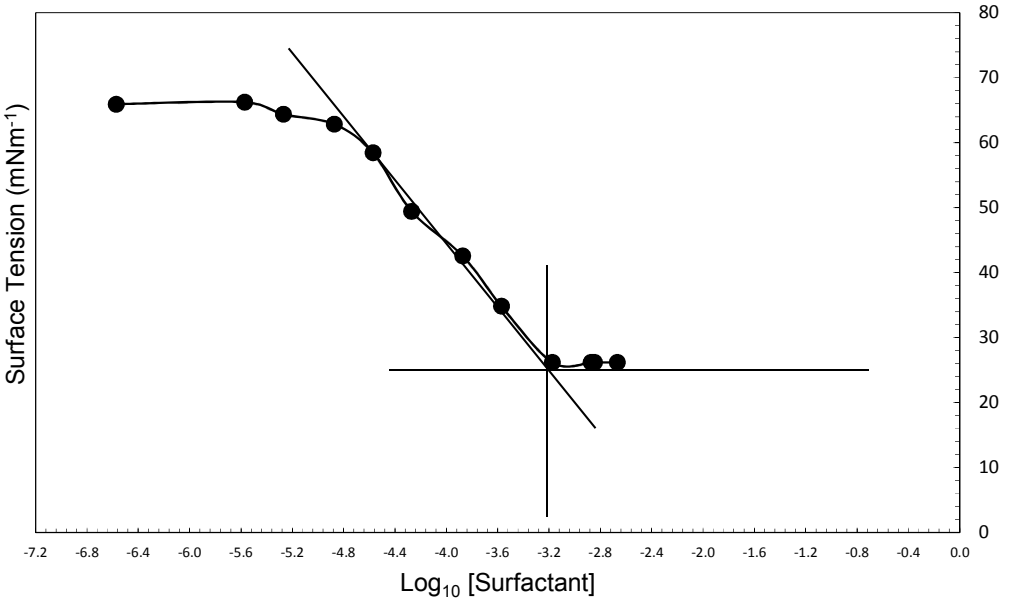


Figure 4.17: Air-water surface tension as a function of the logarithm of surfactant concentration for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside**.**

From the plot in Figure 4.17, the CAC for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was estimated as 5.2 x 10^{-4} mM (antilog of -3.28). Further increase of surfactant concentration results in formation of self-assembled micelles. Because of this, the CAC is more precisely termed as critical micelle concentration (CMC), The surface tension of at the CMC (γ_{cmc}) of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was recorded as 26 mN m^{-1}. This value of γ_{cac} was found equivalent to the minimum surface tension (γ_{min}).

From the plot of air/water surface tension against log concentration of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, the change in the surface tension of air/water in presence of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside can be calculated through the gradient ($d\gamma/d \log C$) of the linear portion of the curve. The gradient was calculated to be -22 mN m^{-1}. Therefore, it can be said that the active reduction in air/water surface tension in the presence of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside is 22 mN m^{-1} or 0.022 J m^{-2}.

It is known that activity of dissolved surfactant in very dilute solution can be replaced by its concentration. By using concentration of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside as a replacement to the chemical potential of surfactant, further explanation on the effect in presence of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside on air/water surface activity can be deduced. Equilibrium between surfactant molecules at the surface of the solution and those in the bulk solution can be expressed through calculation of surface excess of the surfactant using Gibbs equation (Lange & Jeschke, 1987). This provides information on the difference between the amount of surfactant on the surface and those that is actually present in the system (bulk) (Mitropoulos (2008)) thus allowing quantification of maximum adsorption density (Γ_{max}) in mol m^{-2} (Drummond & Wells, 1998). The equation for calculation of the surface excess is:

$$\Gamma_{max}\ (mol\ m^{-2}) = -\frac{1}{RT} \times \frac{d\gamma}{d \log C}$$

$$= -\frac{1}{2.303 \times R \times T} \times \frac{d\gamma}{d \log C}$$

$$= -\frac{1}{2.303 \times R \times T} \times 22.41 \times 10^{-3} \quad \text{(Equation 4.3)}$$

where R is universal gas constant (8.314 J mol^{-1} K), T is temperature (K), γ is the surface tension (N m^{-1}) and C is the concentration of surfactant (mol L^{-1}). With 328.15 K as the working temperature, the value of Γ can be calculated using Equation (4.4) as follows:

$$\Gamma\ (mol\ m^{-2}) = -\frac{1}{2.303 \times 8.31 \times 328.15} \times (-22.41) \times 10^{-3}$$

$$= 4 \times 10^{-6}$$

(Equation 4.4)

The mechanism of surfactant adsorption on the surface of water was said to be monolayer (Soultani *et al.*, 2003). Thus, with information on the value of Γ, calculation on minimum area per surfactant molecule at air/ water surface (A_0) in Å can be subsequently calculated (Equation 4.5)

$$A\left(\text{Å}^2\ per\ molecule\right) = \frac{10^{20}}{N_A \times \Gamma} \qquad \text{(Equation 4.5)}$$

where N_A is the Avogadro constant (6.023×10^{23} mol^{-1}).

Thus,

$$A = \frac{10^{20}}{6.023 \times 10^{23} \times (3.57 \times 10^{-6})} \qquad \text{(Equation 4.6)}$$

$$= 47\ \text{Å}^2\ per\ molecule$$

From the calculation (Equation 4.6), it was found that the minimum area occupied per molecule of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside at air/water surface obtained was 47Å^2.

Energy required for the surfactant in the process of aggregation can be calculated using Gibbs free energy of adsorption by utilizing the value of CAC of the surfactant (methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside). This also known as Gibbs free energy of micellization (ΔG_{mic}) which can be calculated using Equation (4.7) (Rosen, 1978).

$$\Delta G_{mic}\ (kJ\ mol^{-1}) = R \times T \times \ln\left(\frac{CAC}{molarity_{solvent}}\right) \qquad \text{(Equation 4.7)}$$

By replacing the solvent with water (aqueous system), the molarity of water (55.5 mol/l) can be used for calculation. It was found that the critical micelle concentration for methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was 5.2 x 10^{-7} mol L^{-1}. Thus,

$$\Delta G_{mic}\ (kJ\ mol^{-1}) = RT\ \ln\ (\frac{5.2\ \times\ 10^{-7}}{55.5})$$

$$= -\ 50\ \text{kJ mol}^{-1} \qquad \text{(Equation 4.8)}$$

Therefore, it can be said that an aqueous system containing methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside as the surfactant requires 50 kJ amount of energy per moles of methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside in order to reorganize among them to form a self - assembled aggregation.

CHAPTER FIVE

5.0 CONCLUSIONS

In this study, lipase catalyzed synthesis of carbohydrate esters with different fatty acids varying in chain length was performed. The biocatalysis requires a microaqueous system in order for the lipase to drive the esterification. The study utilized one particular hydrophilic head, *i.e.* methyl α-*D*-glucopyranoside, and three different fatty acids [hexadecanoic acid (C-16), tetradecanoic acid (C-14) and dodecanoic acid (C-12)] as the hydrophobic tail. From the studies on the esterification system as well as the physico-chemical analyses on the synthesized carbohydrate esters, it was found that:

1. The activity of enzyme lipase was found to be stable in the organic solvent within the condition applied, as the activity increased up to 60 °C. The efficiency of esterification however was limited by the solubility of the acyl acceptor group (methyl α-*D*-glucopyranoside) in the reaction medium.

2. In a full factorial experimental design only the range of temperature applied for the esterification process was found statistically insignificant ($\alpha = 0.05$) towards the synthesis.

3. All invstigated carbohydrate esters showed melting points below 100 °C. No liquid crystalline phase could be obtained above the melting point, but metastable liquid crystalline phases, which were identified as smectic in one case, were observed below the melting point.

4. All carbohydrate esters synthesized (methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside, methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside and methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside) are stable up to 170 °C and above.

5. The highest water content in a quaternary system of carbohydrate ester/*n*-butanol/*n*-hexadecane/water could be achieved with methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside (52 %) while methyl 6-*O*-tetradecanoyl-α-*D*-glucopyranoside only enabled a water content of up to 34 %. The construction of a pseudo-ternary phase diagram with methyl 6-*O*-hexadecanoyl-α-*D*-glucopyranoside was impossible, because of immediate formation of gels.

6. Methyl 6-*O*-dodecanoyl-α-*D*-glucopyranoside was found to be the best carbohydrate ester to be utilized in water-rich environment as it was the only carbohydrate ester synthesized managed to dissolve in pure water (absence of co- solvent) up to its critical aggregation concentration.

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organogels: effect of temperature and substrate diffusion. *Biotechnology letters, 30*(9), 1627-1631. doi: 10.1007/s10529-008-9734-1

LIST OF PRESENTATIONS AND PUBLICATIONS

PRESENTATIONS

1) Effects of Selected Process Variables On Lipase - Catalyzed 6-*O*-α-Methylglucosyl Hexadecanoate Synthesis. The 3rd International Biotechnology and Biodiversity Conference & Exhibition (2012).
2) Seminar on Physico-Chemical Properties of Lipase Biocatalysed Methyl-6-*0*-hexadecanoyl-α-*D*-Glucopyranoside (University of Malaya, 2012).

PUBLICATIONS

1) Jin, H., Annuar, M.S.M., **Ariffin, M.F.K.,** Gumel, A.M., Ibrahim, S., Heidelberg, T., Bakar, B., Hossain A.B.M.S., and Sharifuddin Y., (2011). **Lipase - Catalyzed Synthesis of 6-*O*-*d*-Glucosyldecanoate in *tert*-butanol: Reaction Optimization and Effect of Mixing Power Input.** Biotechnology and Biotechnology Equipment, (Volume 25), Number 4, DOI: 10.5504/bbeq.2011.0092 (*ISI- cited publication*).
2) Ariffin, M.F.K[1]., Annuar, M.S.M.[1,*], Heidelberg, T[2]., (2013). **Surfactant properties of methyl-α-*D*-glucopyranoside esterified with selected aliphatic carboxylic acids by lipase.** Journal of Surfactant and Detergent (*ISI- cited publication*). SUBMITTED.

PROCEEDING

1) M.F.K Ariffin, M.S.M Annuar and T. Heidelberg, **Effects of Selected Process Variables On Lipase - Catalyzed 6-*O*-α-Methylglucosyl Hexadecanoate Synthesis**. The 3rd International Biotechnology and Biodiversity Conference & Exhibition (BIOJOHOR 2012), June 9-11, 2012, Johor Bahru, Malaysia. ISBN: 978-983-44324-2-9.